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<p>(51) International Patent Classification <sup>6</sup> : C12N 15/12, C07K 14/715, A61K 38/17, C12N 15/62, G01N 33/50, C07K 16/28, A01K 67/027, C12N 5/12</p>	<p>A1</p>	<p>(11) International Publication Number: WO 98/58062 (43) International Publication Date: 23 December 1998 (23.12.98)</p>
<p>(21) International Application Number: PCT/US98/12456 (22) International Filing Date: 12 June 1998 (12.06.98) (30) Priority Data: 08/878,168 18 June 1997 (18.06.97) US (71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US). (72) Inventors: ASHKENAZI, Avi, J.; 1456 Terrytown Street, San Mateo, CA 94401 (US). BAKER, Kevin, P.; 1115 South Grant Street, San Mateo, CA 94401 (US). CHUNTHARA-PAI, Anan; 826 Ellis Drive, Colma, CA 94015 (US). GURNEY, Austin; 1 Debbie Lane, Belmont, CA 94002 (US). KIM, Kyung, Jin; 622 Benvenue Avenue, Los Altos, CA 94024 (US). WOOD, William, I.; 1400 Terrytown Street, San Mateo, CA 94402 (US). (74) Agents: MARSCHANG, Diane, L. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: Apo-2DcR</p> <p>(57) Abstract</p> <p>Novel polypeptides, designated Apo-2DcR, which are capable of binding Apo-2 ligand are provided. Compositions including Apo-2DcR chimeras, nucleic acid encoding Apo-2DcR, and antibodies to Apo-2DcR are also provided.</p>		

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Apo-2DcRFIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2DcR" and to anti-Apo-2DcR antibodies.

BACKGROUND OF THE INVENTIONApoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2

ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- $\alpha$  [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### TNF Family of Receptors

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and

CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 2:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of

engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

#### The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, *Science*, 267:1445 (1995); Chinnaiyan et al., *Science*, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, *Current Biology*, 6:555-562 (1996); Fraser and Evan, *Cell*, 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- $\kappa$ B [Tartaglia et al., *Cell*, 74:845-853 (1993); Hsu et al., *Cell*, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., *supra*; Nagata, *Cell*, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, *Cell*, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., *European Cytokine Network*, 7:Abstr. 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., *Cell*, 81:505-512 (1995); Boldin et al., *J. Biol. Chem.*, 270:387-391 (1995); Hsu et al., *supra*; Chinnaiyan et al., *J. Biol. Chem.*, 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the *Ced-3*-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., *Cell*, 85:803-815 (1996); Muzio et al., *Cell*, 85:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, *supra*].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., *Cell*, 69:597-604 (1992); Tewari et al., *Cell*, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., *Nature*, 375:78-81 (1995); Tewari et al., *J. Biol. Chem.*, 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- $\kappa$ B [Tewari et al., *Curr. Op. Genet. Develop.*, 6:39-44 (1996)]. NF- $\kappa$ B is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., *Genes Develop.*, 9:2723-2735 (1996); Baldwin, *Ann. Rev. Immunol.*, 14:649-681 (1996)]. In

its latent form, NF- $\kappa$ B is complexed with members of the I $\kappa$ B inhibitor family; upon inactivation of the I $\kappa$ B in response to certain stimuli, released NF- $\kappa$ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, *supra*.

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#### SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2DcR." It is believed that Apo-2DcR is a member of the TNFR family; full-length native sequence human Apo-2DcR polypeptide exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Applicants found that Apo-2DcR binds Apo-2 ligand (Apo-2L).

10 In one embodiment, the invention provides isolated Apo-2DcR polypeptide. In particular, the invention provides isolated native sequence Apo-2DcR polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 259 of Figure 1A (SEQ ID NO:1). In other embodiments, the isolated Apo-2DcR polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2DcR polypeptide comprising residues 1 to 259 of Figure 1A (SEQ ID NO:1). Optionally, the  
15 isolated Apo-2DcR polypeptide includes an amino acid sequence comprising residues identified in Figure 1B as -40 to 259 (SEQ ID NO:3). Optionally, the Apo-2DcR polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209087.

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2DcR. Optionally, the isolated extracellular domain sequence comprises amino acid residues 1 to 236  
20 of Fig. 1A (SEQ ID NO:1) or residues 1 to 161 of Fig. 1A (SEQ ID NO:1). Optionally, the isolated extracellular domain sequence comprises an amino acid sequence wherein one or more of the amino acids identified in any of the Apo-2DcR pseudorepeats identified herein (See, Figure 2) have been deleted. Such isolated extracellular domain sequences may include polypeptides comprising a sequence of amino acid residues 1 to X, wherein X is any one of amino acid residues 161 to 236 of Figure 1A (SEQ ID NO:1).

25 In another embodiment, the invention provides chimeric molecules comprising Apo-2DcR polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2DcR fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2DcR fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

30 In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2DcR polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2DcR polypeptide or a particular domain of Apo-2DcR, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

35 (a) the coding region of the nucleic acid sequence of Figure 1A (SEQ ID NO:2) that codes for residue 1 to residue 259 (i.e., nucleotides 193-195 through 967-969), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1A (SEQ ID NO:2) that codes for residue 1 to residue 236 (i.e., nucleotides 193-195 through 898-900), inclusive;

(c) the coding region of the nucleic acid sequence of Figure 1B (SEQ ID NO:4) that codes for residue -40 to residue 259 (i.e., nucleotides 73-75 through 967-969), inclusive;

(d) a sequence corresponding to the sequence of (a), (b) or (c) within the scope of degeneracy of the genetic code.

5 In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2DcR polypeptide or particular domain of Apo-2DcR. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2DcR is further provided.

In another embodiment, the invention provides an antibody which binds to Apo-2DcR. The antibody may be an agonistic, blocking or neutralizing antibody.

10 In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2DcR or Apo-2DcR antibodies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the nucleotide sequence of a native sequence human Apo-2DcR cDNA and its  
15 derived amino acid sequence (initiation site assigned at residue 1 (nucleotides 193-195)).

Figure 1B shows the nucleotide sequence of a native sequence human Apo-2DcR cDNA and its derived amino acid sequence (initiation site assigned at residue -40 (nucleotides 73-75)).

Figure 2 shows the primary structure and mRNA expression of Apo-2 and Apo-2DcR. The figure depicts the deduced amino acid sequences of human Apo-2 and Apo-2DcR aligned with full-length DR4. The  
20 death domain of Apo-2 is aligned with those of DR4, Apo-3/DR3, TNFR1, and CD95; asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., *supra*]. Indicated are the predicted signal peptide cleavage sites (arrows), the two cysteine-rich domains (CRD1, 2) and the transmembrane domain of Apo-2 and DR4 or the hydrophobic C-terminus of Apo-2DcR (underlined). Also indicated are the five potential N-linked glycosylation sites (black boxes) and the five sequence pseudo-repeats (brackets) of  
25 Apo-2DcR.

Figure 3 shows hydropathy plots of Apo-2 and Apo-2DcR. Numbers at the top indicate amino acid positions.

Figure 4 shows binding of radioiodinated Apo-2L to Apo-2DcR-transfected cells and its inhibition by pre-treatment of cells with PI-PLC.

30 Figure 5 shows inhibition of Apo-2L induction of apoptosis by Apo-2DcR.

Figure 6 shows inhibition of Apo-2L activation of NF- $\kappa$ B by Apo-2DcR.

Figure 7A shows expression of Apo-2DcR mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

Figure 7B shows (lack of) expression of Apo-2DcR mRNA in human cancer cell lines as analyzed  
35 by Northern hybridization of human cancer cell line poly A RNA blots.

Figure 8 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.



Figure 9 shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

Figure 10 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

Figure 11 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

Figure 12 shows activation of NF- $\kappa$ B by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF- $\alpha$  and assayed for NF- $\kappa$ B activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 13 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

Figure 14 shows the FACS analysis of Apo-2DcR antibodies (illustrated by the bold lines) as compared to IgG controls (dotted lines). The antibodies (4G3.9.9; 6D10.9.7; and 1C5.24.1 respectively) recognized the Apo-2DcR receptor expressed in HMEC cells.

Figure 15 contains graphs showing results of ELISAs testing binding of Apo-2DcR antibodies 4G3.9.9; 6D10.9.7; and 1C5.24.1 respectively, to Apo-2DcR and to other known Apo-2L receptors referred to as DR4, Apo-2 and DcR2.

Figure 16 is a table providing a summary of isotype and cross-reactivity properties of antibodies 1C5.24.1; 4G3.9.9; and 6D10.9.7.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### I. Definitions

The terms "Apo-2DcR polypeptide" and "Apo-2DcR" when used herein encompass native sequence Apo-2DcR and Apo-2DcR variants (which are further defined herein). These terms encompass Apo-2DcR from a variety of mammals, including humans. The Apo-2DcR may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-2DcR" comprises a polypeptide having the same amino acid sequence as an Apo-2DcR derived from nature. Thus, a native sequence Apo-2DcR can have the amino acid sequence of naturally-occurring Apo-2DcR from any mammal. Such native sequence Apo-2DcR can be isolated from

nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2DcR" specifically encompasses naturally-occurring truncated, secreted, or soluble forms of the Apo-2DcR (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2DcR. In one embodiment of the invention, the native sequence

5 Apo-2DcR is a mature or full-length native sequence Apo-2DcR comprising amino acids 1 to 259 of Fig. 1A (SEQ ID NO:1) or amino acids -40 to 259 of Figure 1B (SEQ ID NO:3). Optionally, the Apo-2DcR polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209087.

The "Apo-2DcR extracellular domain" or "Apo-2DcR ECD" refers to a form of Apo-2DcR which

10 is essentially free of transmembrane and cytoplasmic domains. Ordinarily, Apo-2DcR ECD will have less than 1% of such transmembrane and cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2DcR ECD will comprise amino acid residues 1 to 236 of Fig. 1A (SEQ ID NO:1) or amino acid residues 1 to 161 of Fig. 1A (SEQ ID NO:1). Optionally, the isolated extracellular domain sequence comprises an amino acid sequence wherein one or more of the amino acids identified in any of the

15 Apo-2DcR pseudorepeats identified herein (See, Figure 2) have been deleted. Such isolated extracellular domain sequences may include polypeptides comprising a sequence of amino acid residues 1 to X, wherein X is any one of amino acid residues 161 to 236 of Figure 1A (SEQ ID NO:1).

"Apo-2DcR variant" means a biologically active Apo-2DcR as defined below having at least about 80% amino acid sequence identity with the Apo-2DcR having the deduced amino acid sequence shown in Fig.

20 1A (SEQ ID NO:1) for a full-length native sequence human Apo-2DcR or the sequences identified herein for Apo-2DcR ECD. Such Apo-2DcR variants include, for instance, Apo-2DcR polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1A (SEQ ID NO:1) or the sequences identified herein for Apo-2DcR ECD. Ordinarily, an Apo-2DcR variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence

25 identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1A (SEQ ID NO:1).

"Percent (%) amino acid sequence identity" with respect to the Apo-2DcR sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2DcR sequence, after aligning the sequences and introducing gaps, if necessary, to

30 achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal

35 alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2DcR, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2DcR. The tag polypeptide preferably also is fairly unique so that the antibody does

not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that  
5 has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup  
10 sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2DcR natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2DcR nucleic acid molecule is a nucleic acid molecule that is identified and  
15 separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2DcR nucleic acid. An isolated Apo-2DcR nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2DcR nucleic acid molecules therefore are distinguished from the Apo-2DcR nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2DcR nucleic acid molecule includes Apo-2DcR nucleic acid molecules contained in cells that ordinarily  
20 express Apo-2DcR where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic  
25 cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a  
30 ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

35 The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2DcR monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2DcR antibody compositions with polypeptidic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical

except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2DcR antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein means (1) having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*; (2) having the ability to bind Apo-2 ligand; or (3) having the ability to modulate Apo-2 ligand signaling and Apo-2 ligand activity.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, blastoma, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, neuroblastoma, cervical cancer, ovarian cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial cancer, salivary gland cancer, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, and various types of head and neck cancer.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2DcR polypeptides. In particular, Applicants have identified and isolated various human Apo-2DcR polypeptides. The properties and characteristics of some of these Apo-2DcR polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2DcR polypeptides disclosed herein, it is Applicants' present belief that Apo-2DcR is a member of the TNFR family.

A description follows as to how Apo-2DcR, as well as Apo-2DcR chimeric molecules and anti-Apo-2DcR antibodies, may be prepared.

### A. Preparation of Apo-2DcR

The description below relates primarily to production of Apo-2DcR by culturing cells transformed or transfected with a vector containing Apo-2DcR nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2DcR.

#### 1. Isolation of DNA Encoding Apo-2DcR

The DNA encoding Apo-2DcR may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2DcR mRNA and to express it at a detectable level. Accordingly, human Apo-2DcR DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as libraries of human cDNA described in Example 1. The Apo-2DcR-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2DcR or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as

described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2DcR is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

5 One method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and  
10 include the use of radiolabels like  $^{32}\text{P}$ -labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect  
15 precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Apo-2DcR variants can be prepared by introducing appropriate nucleotide changes into the Apo-2DcR DNA, or by synthesis of the desired Apo-2DcR polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2DcR, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

20 Variations in the native full-length sequence Apo-2DcR or in various domains of the Apo-2DcR described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2DcR that results in a change in the amino acid sequence of the Apo-2DcR as compared with the native sequence Apo-2DcR. Optionally the  
25 variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2DcR molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1982)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos.  
30 Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2DcR variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine,  
35 glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol.

Biol., 105:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2DcR variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2DcR variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2DcR and an Apo-2DcR variant, a convenient one for binding is the dissociation constant  $K_d$  of the complex formed between the Apo-2DcR variant and Apo-2L as compared to the  $K_d$  for the native sequence Apo-2DcR. Generally, a  $\geq 3$ -fold increase or decrease in  $K_d$  per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2DcR with the Apo-2L.

Optionally, representative sites in the Apo-2DcR sequence suitable for mutagenesis (such as deletion of one or more amino acids) would include sites within the extracellular domain, and particularly, within one or more of the cysteine-rich domains or within one or more of the pseudorepeats. Such variations can be accomplished using the methods described above.

## 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding Apo-2DcR may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

### (i) Signal Sequence Component

The Apo-2DcR may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2DcR DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2DcR presequence that normally directs insertion of Apo-2DcR in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2DcR.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2DcR DNA. However, the recovery of genomic DNA encoding Apo-2DcR is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2DcR DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., *J. Molec. Appl. Genet.*, 1:327 (1982)], mycophenolic acid (Mulligan et al., *Science*, 209:1422 (1980)) or hygromycin [Sugden et al., *Mol. Cell. Biol.*, 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2DcR nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the



transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2DcR. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells.

- 5 Increased quantities of Apo-2DcR are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2DcR. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2DcR, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:23 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

35 (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2DcR nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2DcR nucleic

acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2DcR encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2DcR promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2DcR DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:617 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (*trp*) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the *tac* promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2DcR [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2DcR.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2DcR transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock

promoters, and from the promoter normally associated with the Apo-2DcR sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)].  
 5 The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-  
 10 508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon  $\beta$ 1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney  
 15 cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

#### (v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2DcR of this invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting  
 20 elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:464 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin,  
 25 elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2DcR  
 30 coding sequence, but is preferably located at a site 5' from the promoter.

#### (vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the  
 35 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2DcR.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

5 For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

10 (viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2DcR may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2DcR variants.

20 (ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2DcR in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

25 Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2DcR-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, 35 is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2DcR are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of

invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., *Bio/Technology*, 6:47-55 (1988); Miller et al., in *Genetic Engineering*, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature*, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2DcR can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2DcR-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., *J. Mol. Appl. Gen.*, 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., *Tissue Culture*, Academic Press. Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen. Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.*, 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2DcR production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., *supra*, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for

transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-467 (1973) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

#### 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2DcR may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2DcR may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

#### 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes,

including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

- 5           Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent  
10   labels, or luminescent labels.

- Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2DcR polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2DcR DNA and encoding a specific  
15   antibody epitope.

#### 6. Purification of Apo-2DcR Polypeptide

- Forms of Apo-2DcR may be recovered from culture medium or from host cell lysates. If the Apo-2DcR is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage. Apo-2DcR can also  
20   be released from the cell-surface by enzymatic cleavage of its glycopospholipid membrane anchor.

- When Apo-2DcR is produced in a recombinant cell other than one of human origin, the Apo-2DcR is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2DcR from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2DcR. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-  
25   2DcR thereafter is purified from contaminants soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

- 30           Apo-2DcR variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2DcR, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2DcR fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide.  
35   Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native

sequence Apo-2DcR may require modification to account for changes in the character of Apo-2DcR or its variants upon expression in recombinant cell culture.

7. Covalent Modifications of Apo-2DcR Polypeptides

Covalent modifications of Apo-2DcR are included within the scope of this invention. One type of covalent modification of the Apo-2DcR is introduced into the molecule by reacting targeted amino acid residues of the Apo-2DcR with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the Apo-2DcR.

Derivatization with bifunctional agents is useful for crosslinking Apo-2DcR to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2DcR antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2DcR molecules to generate Apo-2DcR dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidylesters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutamyl and asparagyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2DcR polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2DcR, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2DcR.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.



Addition of glycosylation sites to the Apo-2DcR polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2DcR (for O-linked glycosylation sites). The Apo-2DcR amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2DcR polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2DcR polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2DcR polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duksin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2DcR comprises linking the Apo-2DcR polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 8. Apo-2DcR Chimeras

The present invention also provides chimeric molecules comprising Apo-2DcR fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2DcR with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl-terminus of the Apo-2DcR. The presence of such epitope-tagged forms of the Apo-2DcR can be detected using an antibody against the tag polypeptide. Also, provision of the

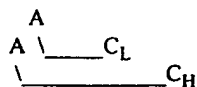
epitope tag enables the Apo-2DcR to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

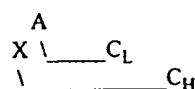
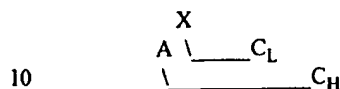
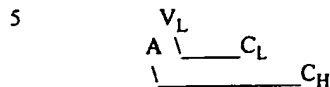
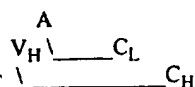
Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:14163-14166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2DcR may be constructed and produced according to the methods described above. Apo-2DcR-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2DcR portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2DcR-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2DcR will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N-terminus or the C-terminus and used as a purification handle in affinity chromatography.

Epitope-tagged Apo-2DcR can be purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2DcR can then be eluted from the affinity column using techniques known in the art.

In another embodiment, the chimeric molecule comprises an Apo-2DcR polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2DcR, such as an extracellular domain sequence of Apo-2DcR fused to an immunoglobulin sequence. This includes chimeras in monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

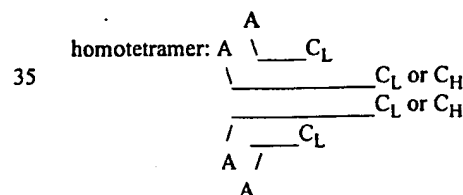
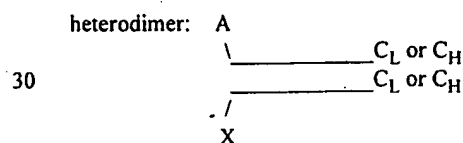
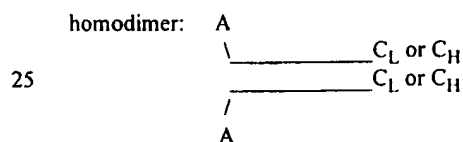


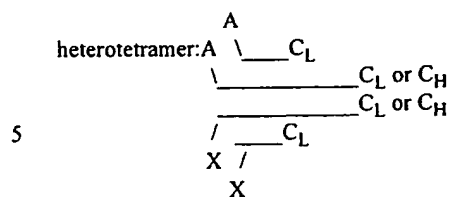


- 15 A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

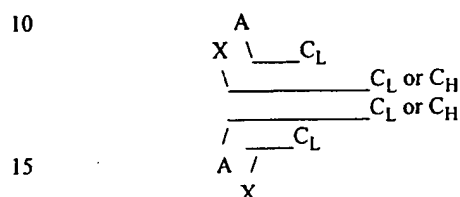
- 20 The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

monomer:  $A \quad C_L \text{ or } C_H$





and



In the foregoing diagrams, "A" means an Apo-2DcR sequence or an Apo-2DcR sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such as pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2DcR sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, *Nature*, 312:597 (13 December 1984); Neuberger et al., *Nature*, 312:604-608 (13 December 1984); Sharon et al., *Nature*, 309:364-367 (24 May 1984); Morrison et al., *Proc. Nat'l. Acad. Sci. USA*, 81:6851-6855 (1984); Morrison et al., *Science*, 229:1202-1207 (1985); Boulianne et al., *Nature*, 312:643-646 (13 December 1984); Capon et al., *Nature*, 337:525-531 (1989); Traunecker et al., *Nature*, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed as follows. The DNA including a region encoding the desired sequence, such as an Apo-2DcR and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2DcR or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native

signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989). Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., *supra*; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, [J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

#### B. Therapeutic and Non-therapeutic Uses for Apo-2DcR

Apo-2DcR, as disclosed in the present specification, can be employed therapeutically to regulate apoptosis and/or NF- $\kappa$ B activation by Apo-2L or by another ligand that Apo-2DcR binds to in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2DcR chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2DcR or the Apo-2DcR immunoadhesin described in the Examples below) comprising immunoglobulin sequences can also be employed therapeutically to inhibit Apo-2L activities, for example, apoptosis or NF- $\kappa$ B induction or the activity of another ligand that Apo-2DcR binds to.

Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the carrier include buffers such as saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7.4 to about 7.8. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration.

Administration to a mammal may be accomplished by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure delivery to the bloodstream in an effective form.

Effective dosages and schedules for administration may be determined empirically, and making such determinations is within the skill in the art.

It is contemplated that other, additional therapies may be administered to the mammal, and such includes but is not limited to, chemotherapy and radiation therapy, immunoadjuvants, cytokines, and antibody-

based therapies. Examples include interleukins (e.g., IL-1, IL-2, IL-3, IL-6), leukemia inhibitory factor, interferons, TGF-beta, erythropoietin, thrombopoietin, and HER-2 antibody. Other agents known to induce apoptosis in mammalian cells may also be employed, and such agents include TNF- $\alpha$ , TNF- $\beta$  (lymphotoxin- $\alpha$ ), CD30 ligand, 4-1BB ligand, and Apo-1 ligand.

5 Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytosine, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. Preparation and dosing schedules for such chemotherapy may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing  
10 schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapy is preferably administered in a pharmaceutically-acceptable carrier, such as those described above.

The Apo-2DcR of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2DcR may be used as a diagnostic for tissue-specific typing. For example,  
15 procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2DcR is present in the cell type(s) being evaluated. Apo-2DcR nucleic acid will also be useful for the preparation of Apo-2DcR by the recombinant techniques described herein.

The isolated Apo-2DcR may be used in quantitative diagnostic assays as a control against which  
20 samples containing unknown quantities of Apo-2DcR may be prepared. Apo-2DcR preparations are also useful in generating antibodies, as standards in assays for Apo-2DcR (e.g., by labeling Apo-2DcR for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

25 Isolated, native forms of Apo-2DcR, such as described in the Examples, may be employed to identify alternate forms of Apo-2DcR; for example, forms that possess cytoplasmic domain(s) which may be involved in signaling pathway(s). Modified forms of the Apo-2DcR, such as the Apo-2DcR-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2DcR antibodies.

Nucleic acids which encode Apo-2DcR or its modified forms can also be used to generate either  
30 transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2DcR or an appropriate sequence  
35 thereof (such as Apo-2DcR-IgG) can be used to clone genomic DNA encoding Apo-2DcR in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2DcR. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2DcR transgene incorporation

with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2DcR introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2DcR. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis.

- 5 In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2DcR such as the Apo-2DcR ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2DcR.

- 10 Alternatively, non-human homologues of Apo-2DcR can be used to construct an Apo-2DcR "knock out" animal which has a defective or altered gene encoding Apo-2DcR as a result of homologous recombination between the endogenous gene encoding Apo-2DcR and altered genomic DNA encoding Apo-2DcR introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2DcR can be used to clone genomic DNA encoding Apo-2DcR in accordance with established techniques. A portion of the  
15 genomic DNA encoding Apo-2DcR can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, *Cell*, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously  
20 recombined with the endogenous DNA are selected [see e.g., Li et al., *Cell*, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the  
25 homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2DcR polypeptide, including for example, development of tumors.

30 C. Anti-Apo-2DcR Antibody Preparation

The present invention further provides anti-Apo-2DcR antibodies. Antibodies against Apo-2DcR may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

- 35 The Apo-2DcR antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2DcR polypeptide or a fusion

protein thereof. An example of a suitable immunizing agent is a Apo-2DcR-IgG fusion protein or chimeric molecule (including an Apo-2DcR ECD-IgG fusion protein). Cells expressing Apo-2DcR at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include  
5 but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the  
10 serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

## 2. Monoclonal Antibodies

The Apo-2DcR antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and  
15 Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2DcR polypeptide or a fusion protein thereof.  
20 An example of a suitable immunizing agent is a Apo-2DcR-IgG fusion protein or chimeric molecule. A specific example of an immunogen is described in Example 13 below. Cells expressing Apo-2DcR at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as  
25 polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the  
30 parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium.  
35 More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al.,



Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2DcR. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

As described in the Examples below, anti-Apo-2DcR monoclonal antibodies have been prepared. Several of these antibodies, referred to as 4G3.9.9, 6D10.9.7, and 1C5.24.1 have been deposited with ATCC and have been assigned deposit accession numbers \_\_\_\_\_, \_\_\_\_\_, and \_\_\_\_\_, respectively. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as one or more of the antibodies secreted by the hybridoma cell lines deposited under accession numbers \_\_\_\_\_, \_\_\_\_\_, or \_\_\_\_\_. The term "biological characteristics" is used to refer to the *in vitro* and or *in vivo* activities or properties of the monoclonal antibodies, such as the ability to bind to Apo-2DcR or to substantially block, induce, or enhance Apo-2DcR activation. Optionally, the monoclonal antibody will bind to the same epitope as at least one of the three antibodies specifically referred to above. Such epitope binding can be determined by conducting various assays, like those described herein and in the examples.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

*In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain ( $CH_1$ ) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain  $CH_1$  domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2DcR antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab',  $F(ab')_2$  or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an

"import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immunol., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1992); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al.,

Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)).

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2DcR, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/20373; EP 03089]. It is contemplated that the antibodies

may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

5 D. Therapeutic and Non-therapeutic Uses for Apo-2DcR Antibodies

The Apo-2DcR antibodies of the invention have therapeutic utility. For example, Apo-2DcR antibodies which cross-react with other receptors for Apo-2 ligand may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects. Optionally, Apo-2DcR blocking antibodies can be used in combination with an Apo-2 ligand treatment. Such  
10 Apo-2DcR antibodies can block the Apo-2DcR receptor, and increase bioavailability of the administered Apo-2 ligand. Therapeutic compositions and modes of administration (such as described above for Apo-2DcR) may be employed.

Apo-2DcR antibodies may further be used in immunohistochemistry staining assays or diagnostic assays for Apo-2DcR, *e.g.*, detecting its expression in specific cells, tissues, or serum. Various  
15 diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety  
20 may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 194:495 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and  
25 Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2DcR antibodies also are useful for the affinity purification of Apo-2DcR from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2DcR are immobilized on a suitable support, such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2DcR to be purified, and thereafter the support is washed  
30 with a suitable solvent that will remove substantially all the material in the sample except the Apo-2DcR, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2DcR from the antibody.

E. Kits Containing Apo-2DcR or Apo-2DcR Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits  
35 containing Apo-2DcR or Apo-2DcR antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active

agent in the composition is Apo-2DcR or an Apo-2DcR antibody. The label on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other  
5 containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by  
10 reference in their entirety.

### EXAMPLES

All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those  
15 cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

#### EXAMPLE I

##### Isolation of cDNA clones Encoding Human Apo-2DcR

###### 1. Preparation of oligo dT primed cDNA library ("LIB111")

mRNA was isolated from human breast carcinoma tissue using reagents and protocols from  
20 Invitrogen, San Diego, CA (Fast Track 2). This RNA was used to generate an oligo dT primed cDNA library ("LIB111") in the vector pRK5D using reagents and protocols from Life Technologies, Gaithersburg, MD (Super Script Plasmid System). In this procedure, the double stranded cDNA was sized to greater than 1000 bp and the SalI/NotI linked cDNA was cloned into XhoI/NotI cleaved vector. pRK5D is a cloning vector  
25 that has an sp6 transcription initiation site followed by an SfiI restriction enzyme site preceding the XhoI/NotI cDNA cloning sites.

###### 2. Preparation of random primed cDNA library ("LIB118")

A secondary cDNA library was generated in order to preferentially represent the 5' ends of the primary cDNA clones. Sp6 RNA was generated from the primary library (LIB111, described above), and  
30 this RNA was used to generate a random primed cDNA library ("LIB118") in the vector pSST-AMY.0 using reagents and protocols from Life Technologies (Super Script Plasmid System, referenced above). In this procedure the double stranded cDNA was sized to 500-1000 bp, linked with blunt to NotI adaptors, cleaved with SfiI, and cloned into SfiI/NotI cleaved vector. pSST-AMY.0 is a cloning vector that has a yeast alcohol dehydrogenase promoter preceding the cDNA cloning sites and the mouse amylase sequence (the mature  
35 sequence without the secretion signal) followed by the yeast alcohol dehydrogenase terminator, after the cloning sites. Thus, cDNAs cloned into this vector that are fused in frame with amylase sequence will lead to the secretion of amylase from appropriately transfected yeast colonies.

### 3. Transformation and Detection

DNA from LIB118 was chilled on ice to which was added electrocompetent DH10B bacteria (Life Technologies, 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Technologies, 1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient. The purified DNA was then carried on to the yeast protocols below.

The yeast methods employed in the present invention were divided into three categories: (1) Transformation of yeast with the plasmid/cDNA combined vector; (2) Detection and isolation of yeast clones secreting amylase; and (3) PCR amplification of the insert directly from the yeast colony and purification of the DNA for sequencing and further analysis.

While any yeast strain containing a stable mutant *ura3* is useable with the present invention, the preferable yeast strain used with the practice of the invention was HD56-5A (ATCC-90785). This strain had the following genotype: MAT alpha, *ura3-52*, *leu2-3*, *leu2-112*, *his3-11*, *his3-15*, MAL<sup>+</sup>, SUC<sup>+</sup>, GAL<sup>+</sup>.

Transformation was performed based on the protocol outlined by Gietz et al., Nucl. Acid. Res., 20:1425 (1992). With this procedure, we obtained transformation efficiencies of approximately  $1 \times 10^5$  transformants per microgram of DNA. Transformed cells were then inoculated from agar into YEPD complex media broth (100 ml) and grown overnight at 30°C. The YEPD broth was prepared as described in Kaiser et al., Methods in Yeast Genetics, Cold Spring Harbor Press, Cold Spring Harbor, N.Y, USA, p. 207 (1994). The overnight culture was then diluted to about  $2 \times 10^6$  cells/ml (approx. OD<sub>600</sub>=0.1) into fresh YEPD broth (500 ml) and regrown to  $1 \times 10^7$  cells/ml (approx. OD<sub>600</sub>=0.4-0.5). This usually took about 3 hours to complete.

The cells were then harvested and prepared for transformation by transfer into GS3 rotor bottles in a Sorval GS3 rotor at 5,000 rpm for 5 minutes, the supernatant discarded, and then resuspended into sterile water, and centrifuged again in 50 ml falcon tubes at 3,500 rpm in a Beckman GS-6KR centrifuge. The supernatant was discarded and the cells were subsequently washed with LiAc/TE (10 ml, 10 mM Tris-HCl, 1 mM EDTA pH 7.5, 100 mM Li<sub>2</sub>OOCCH<sub>3</sub>), and resuspended into LiAc/TE (2.5 ml).

Transformation took place by mixing the prepared cells (100 µl) with freshly denatured single stranded salmon testes DNA (Lofstrand Labs, Gaithersburg, MD, USA) and transforming DNA (1 µg, vol. < 10 µl) in microfuge tubes. The mixture was mixed briefly by vortexing, then 40% PEG/TE (600 µl, 40% polyethyleneglycol-4000, 10 mM Tris-HCl, 1 mM EDTA, 100 mM Li<sub>2</sub>OOCCH<sub>3</sub>, pH 7.5) was added. This mixture was gently mixed and incubated at 30°C while agitating for 30 minutes. The cells were then heat shocked at 42°C for 15 minutes, and the reaction vessel centrifuged in a microfuge at 12,000 rpm for 5-10 seconds, decanted and resuspended into TE (500 µl, 10 mM Tris-HCl, 1 mM EDTA pH 7.5) followed by recentrifugation. The cells were then diluted into TE (1 ml) and aliquots (200 µl) were spread onto the selective media previously prepared in 150 mm growth plates (VWR).

Alternatively, instead of multiple small reactions, the transformation was performed using a single, large scale reaction, wherein reagent amounts were scaled up accordingly.

The selective media used was a synthetic complete dextrose agar lacking uracil (SCD-Ura) prepared as described in Kaiser et al., Methods in Yeast Genetics, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., USA, p. 208-210 (1994). Transformants were grown at 30°C for 2-3 days.

The detection of colonies secreting amylase was performed by including red starch in the selective growth media. Starch was coupled to the red dye (Reactive Red-120, Sigma) as per the procedure described by Biely et al., Anal. Biochem., 172:176-179 (1988). The coupled starch was incorporated into the SCD-Ura agar plates at a final concentration of 0.15% (w/v), and was buffered with potassium phosphate to a pH of 7.0 (50-100 mM final concentration).

The positive colonies were picked and streaked across fresh selective media (onto 150 mm plates) in order to obtain well isolated and identifiable single colonies. This step also ensured maintenance of the plasmid amongst the transformants. Well isolated single colonies positive for amylase secretion were detected by direct incorporation of red starch into buffered SCD-Ura agar. Positive colonies were determined by their ability to break down starch resulting in a clear halo around the positive colony visualized directly.

#### 4. Isolation of DNA by PCR Amplification

When a positive colony was isolated, a portion of it was picked by a toothpick and diluted into sterile water (30 µl) in a 96 well plate. At this time, the positive colonies were either frozen and stored for subsequent analysis or immediately amplified. An aliquot of cells (5 µl) was used as a template for the PCR reaction in a 25 µl volume containing: 0.5 µl KlenTaq (Clontech, Palo Alto, CA); 4.0 µl 10 mM dNTP's (Perkin Elmer-Cetus); 2.5 µl Kentaq buffer (Clontech); 0.25 µl forward oligo 1; 0.25 µl reverse oligo 2; 12.5 µl distilled water. The sequence of the forward oligonucleotide 1 was:

TGTAACGACGCGCCAGTTAAATAGACCTGCAATTATTAATCT  
[SEQ ID NO:5]

The sequence of reverse oligonucleotide 2 was:

CAGGAAACAGCTATGACCACCTGCACACCTGCAAAATCCATT  
[SEQ ID NO:6]

PCR was then performed as follows:

- |    |    |                       |                  |
|----|----|-----------------------|------------------|
|    | a. | Denature              | 92°C, 5 minutes  |
| 30 | b. | 3 cycles of Denature  | 92°C, 30 seconds |
|    |    | Anneal                | 59°C, 30 seconds |
|    |    | Extend                | 72°C, 60 seconds |
|    | c. | 3 cycles of Denature  | 92°C, 30 seconds |
|    |    | Anneal                | 57°C, 30 seconds |
| 35 |    | Extend                | 72°C, 60 seconds |
|    | d. | 25 cycles of Denature | 92°C, 30 seconds |
|    |    | Anneal                | 55°C, 30 seconds |
|    |    | Extend                | 72°C, 60 seconds |
|    | e. | Hold                  | 4°C              |



The underlined regions of the oligonucleotides annealed to the ADH promoter region and the amylase region, respectively, and amplified a 307 bp region from vector pSST-AMY.0 when no insert was present. Typically, the first 18 nucleotides of the 5' end of these oligonucleotides contained annealing sites for the sequencing primers. Thus, the total product of the PCR reaction from an empty vector was 343 bp. However, signal sequence-fused cDNA resulted in considerably longer nucleotide sequences.

Following the PCR, an aliquot of the reaction (5 µl) was examined by agarose gel electrophoresis in a 1% agarose using a Tris-Borate-EDTA (TBE) buffering system as described by Sambrook et al., *supra*. Clones resulting in a single strong PCR product larger than 400 bp were further analyzed by DNA sequencing after purification with a 96 Qiaquick PCR clean-up column (Qiagen Inc., Chatsworth, CA).

#### 10 5. Identification of Full-length Clone

A cDNA sequence ("DNA21705") isolated in the above screen was found to have certain amino acid sequence similarity or homology with human TNFR1:

TNFR1	81	CRECESG-SFTASENHLRHCLSCSKCRKEMG
		* * * * *
15 DNA21705	164	CNPCTEGVDYTNASNNEPSCFPCTVCKSD--

QVEISSCTVDRDTCVGCGRK (SEQ ID NO:7)

\* \* \* \* \*

QKHKSCTMTRDTCVCCKE (SEQ ID NO:8)

Based on the similarity, probes were generated from the sequence of DNA21705 and used to screen a human fetal lung library ("LIB25") prepared as described in paragraph 1 above. The cloning vector was pRK5B (pRK5B is a precursor of pRK5D that does not contain the SfiI site), and the cDNA size cut was less than 2800 bp. A full length clone was identified (DNA33085) (pRK5-hApo-2DcR) (also referred to as Apo2-DcR deposited as ATCC 209087, as indicated below) that contained a single open reading frame with an apparent translational initiation site at nucleotide positions 193-195 [Kozak et al., *supra*] and ending at the stop codon found at nucleotide positions 970-972 (Fig. 1A; SEQ ID NO:2). The predicted polypeptide precursor is 259 amino acids long and has a calculated molecular weight of approximately 27.4 kDa. Sequence analysis indicated an N-terminal signal peptide, two cysteine-rich domains, a sequence that contains four nearly identical 15 amino acid tandem repeats, and a hydrophobic C-terminal region. (Figures 2 and 3). The hydrophobic sequence at the C-terminus is preceded by a pair of small amino acids (Ala223 and Ala224); this structure and the absence of an apparent cytoplasmic domain suggests that Apo-2DcR may be a glycosylphosphatidylinositol (GPI) anchored protein [see, Moran, *J. Biol. Chem.*, 266:1250-1257 (1991)]. Apo-2DcR contains five potential N-linked glycosylation sites. (Fig. 2)

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4 and Apo-2 (described further below), Apo-2DcR contains two extracellular cysteine-rich pseudorepeats (Fig. 2), whereas other

identified mammalian TNFR family members contain three or more such domains [Smith et al., *Cell*, 76:959 (1994)].

Based on an alignment analysis of the full-length sequence shown in Figure 1A (SEQ ID NO:1), Apo-2DcR shows more sequence identity to DR4 (60%) and Apo-2 (50%) than to other apoptosis-linked receptors, such as Apo-3, TNFR1, or Fas/Apo-1.

In Fig. 1B, Applicants have shown that the apparent translational initiation site may alternatively be assigned at nucleotide positions 93-95 (identified in Fig. 1B as amino acid residue -40; SEQ ID NO:4). The Apo-2DcR shown in Fig. 1B includes amino acid residues -40 to 259.

#### EXAMPLE 2

##### 10 Binding of Apo-2DcR to Apo-2L and Effect of PI-PLC on Apo-2DcR Activity

To test whether Apo-2DcR binds to Apo-2L, and to assess whether Apo-2DcR is GPI-linked, binding of radioiodinated Apo-2L to Apo-2DcR-transfected 293 cells was analyzed. The effect of pre-treatment of the cells with phosphatidylinositol-specific phospholipase C (PI-PLC) on the binding was also analyzed.

Human 293 cells (ATCC CRL 1573) were plated in 100mm plates ( $1 \times 10^6$  cells/plate) and transfected with 20  $\mu$ g/plate pRK5 or pRK5 encoding the full-length Apo-2DcR (described in Example 1, ATCC deposit 209087) using calcium phosphate precipitation. After 24 hours, the cells were harvested in PBS/10 mM EDTA, washed in phosphate buffered saline (PBS), resuspended in 2 ml PBS per original plate and divided into two 1 ml aliquots per transfection. PI-PLC [Treanor et al., *Nature*, 382:80-83 (1996)] (1  $\mu$ g/ml) was added to one of the two aliquots derived from each transfection, and the cells were incubated 1 hour at 37° C. The cells were washed and resuspended in 1 ml PBS containing 1% BSA (Sigma), and 0.04 ml aliquots were placed into tubes in triplicate. To these tubes was added approximately 20,000cpm  $^{125}$ I-Apo-2L (Apo-2L is described in Pitti et al., *supra*, and was radioiodinated by conventional lactoperoxidase methodology) in 0.005ml, along with 0.005 ml PBS, or 0.005  $\mu$ l unlabeled Apo-2L in PBS (final concentration 0.5  $\mu$ g/ml) for determination of nonspecific binding. After a 1 hour incubation at room temperature, the cells were washed in ice cold PBS, pelleted, and counted for radioactivity.

Transfection by Apo-2DcR led to a marked increase in the amount of specific Apo-2L binding, indicating that Apo-2DcR binds Apo-2L (Fig. 4). Treatment with PI-PLC caused a marked reduction in Apo-2L binding, indicating that Apo-2DcR is a GPI-anchored receptor (Fig. 4).

#### 30 EXAMPLE 3

##### Inhibition of Apo-2L Function by Full-length Apo-2DcR

The absence of a cytoplasmic region in Apo-2DcR suggested that this receptor is involved in modulation, rather than in transduction of Apo-2L signaling. Thus, the effect of Apo-2DcR transfection on cellular responsiveness to Apo-2L was examined.

Human 293 cells, which express both DR4 and Apo-2 mRNA (data not shown), were plated in 100mm plates ( $1 \times 10^6$  cells/plate) and transfected with 3  $\mu$ g per plate pRK encoding green fluorescent protein (GFP; purchased from Clontech) together with 20  $\mu$ g/plate pRK5 or pRK5-hApo-2DcR (see Example 2) using calcium phosphate precipitation. After 18 hours, the cells were treated with PBS or with Apo-2L (Pitti et al., *supra*, 0.5  $\mu$ g/ml) and examined over 6 hours under a fluorescence microscope equipped with Hoffman optics

(which enables clear viewing of non-fixed cells on plastic). GFP-positive cells were identified by green fluorescence and scored for apoptosis by morphologic criteria such as membrane blebbing and cytoplasmic condensation.

Transfection by Apo-2DcR markedly reduced responsiveness to Apo-2L as measured by apoptosis induction (Fig. 5).

In a similar experiment, the 293 cells were transfected by pRK5 or pRK5-hApo-2DcR (20  $\mu$ g/plate) and analyzed 18 hours later for activation of NF- $\kappa$ B by Apo-2L (0.5  $\mu$ g/ml), as in Example 10 below. The results showed that Apo-2DcR inhibits Apo-2L function as measured by apoptosis induction as well as by NF- $\kappa$ B activation (Fig. 6).

#### EXAMPLE 4

##### Northern Blot Analysis

Expression of Apo-2DcR mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 1.2 kilobase  $^{32}$ P-labelled DNA probe based on the full length Apo-2DcR cDNA; the probe was generated by digesting the pRK5-Apo-2DcR plasmid with EcoRI and purifying the Apo-2DcR cDNA insert. Human fetal RNA blot MTN (Clontech), human adult RNA blot MTN-II (Clontech) and human cancer cell line RNA blot (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure by phosphorimager analysis (Fuji).

As shown in Fig. 7A, several Apo-2DcR mRNA transcripts were detected. Relatively high expression was seen in adult peripheral blood leukocytes (PBL), spleen, lung, liver and placenta. Some adult tissues that express Apo-2DcR, e.g., PBL and spleen, have been shown to express Apo-2 (Example 11 below) and DR4 [Pan et al., *supra*].

As shown in Fig. 7B, the Apo-2DcR message is absent from most of the human tumor cell lines examined (namely, HL60 promyelocytic leukemia, HeLa S3 cervical carcinoma, K562 chronic myelogenous leukemia, MOLT4 acute lymphoblastic leukemia, SW480 colorectal adenocarcinoma, A549 lung carcinoma, and G361 melanoma), and particularly the approximate 1.5 kB transcript which corresponds in size to the Apo-2DcR cDNA. The apparent expression of Apo-2DcR in the above-mentioned normal human tissues but not the identified tumor cell types suggests that the Apo-2DcR receptor may allow for preferential killing of cancer cells by Apo-2 ligand (possibly through protection of normal cells but not cancerous cells).

#### EXAMPLE 5

##### Isolation of cDNA clones Encoding Human Apo-2

An expressed sequence tag (EST) DNA database (LIFESEQ<sup>TM</sup>, Incyte Pharmaceuticals, Palo Alto, CA) was searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., *Curr. Biol.*, 6:750 (1996)]. Human pancreas ("LIB55") and human kidney ("LIB28") cDNA libraries (prepared as described in Example 1 above in pRK5 vectors), were screened by hybridization with a synthetic oligonucleotide probe:

GGGAGCCGCTCAT GAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAG  
GCAGCGGG (SEQ ID NO:9) based on the EST.

Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for codon 410 (using the numbering system for Fig. 8); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

The entire nucleotide sequence of Apo-2 is shown in Figure 8 (SEQ ID NO:10). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC 209021, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., *supra*] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 8; SEQ ID NO:10). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 9; SEQ ID NO:11). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.

Like DR4 and Apo-2DcR, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 9), whereas other identified mammalian TNFR family members contain three or more such domains [Smith et al., *Cell*, 76:959 (1994)].

The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 8; see also Fig. 2) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., *supra*] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2).

Based on an alignment analysis (using the ALIGN computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

#### EXAMPLE 6

##### A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 8) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 8 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 7) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

### B. Expression of Apo-2 ECD as an Immunoaderhin

A soluble Apo-2 ECD immunoaderhin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 8) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., *Proc. Natl. Acad. Sci.*, **88**:10535-10539(1991)]. The immunoaderhin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., *supra*.

### EXAMPLE 7

#### Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 6 above) (5 ml) were incubated with 5  $\mu$ g poly-histidine-tagged soluble Apo-2L [Pitti et al., *supra*] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25  $\mu$ l anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4<sup>o</sup> C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2  $\mu$ g/ml) as described in Marsters et al., *J. Biol. Chem.*, **272**:14029-14032 (1997).

The results, shown in Figure 10, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 6) and then analyzing the samples on a BIACORE<sup>TM</sup> instrument. The BIACORE<sup>TM</sup> analysis indicated a dissociation constant ( $K_d$ ) of about 1 nM. BIACORE<sup>TM</sup> analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., *Nature*, **312**:724 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

### EXAMPLE 8

#### Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., *supra*, Nagata et al., *supra*]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 11A); DNA fragmentation (Fig. 11B); or by FACS analysis of

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phosphatidylserine exposure (Fig. 11C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 11A and 11B, the Apo-2 transfected 293 cells underwent marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200  $\mu$ M at the time of transfection. As shown in Fig. 11C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al., supra] did not inhibit apoptosis induction by Apo-2 when co-transfected into HeLa cells with Apo-2 (Fig. 11C). These results suggest that Apo-2 signals apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

EXAMPLE 9Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5  $\mu$ g/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5  $\mu$ g/ml) together with anti-Flag antibody (Sigma) (1  $\mu$ g/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 11D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 11D), confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 11E). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 11E).

EXAMPLE 10Activation of NF- $\kappa$ B by Apo-2

An assay was conducted to determine whether Apo-2 activates NF- $\kappa$ B.

HeLa cells were transfected with pRK5 expression plasmids encoding full-length native sequence Apo-2, DR4 or Apo-3 and harvested 24 hours after transfection. Nuclear extracts were prepared and 1  $\mu$ g of nuclear protein was reacted with a  $^{32}$ P-labelled NF- $\kappa$ B-specific synthetic oligonucleotide probe ATCAGGGACTTTCGCTGGGGACTTTCG (SEQ ID NO:12) [see, also, MacKay et al., J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant  $^{32}$ P-labelled synthetic oligonucleotide AGGATGGGAAGTGTGTGATATCCTTGAT (SEQ ID NO:13). In some samples, antibody to p65/RelA subunits of NF- $\kappa$ B (1  $\mu$ g/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 12. As shown in Fig. 12A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF- $\kappa$ B activation as measured by the electrophoretic mobility shift assay; the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF- $\kappa$ B inhibited the mobility of the NF- $\kappa$ B probe, implicating p65 in the response to all 3 receptors.

5 An assay was also conducted to determine if Apo-2L itself can regulate NF- $\kappa$ B activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., *supra*) or TNF-alpha (Genentech, Inc., see Pennica et al., *Nature*, 312:724 (1984)) (1  $\mu$ g/ml) and assayed for NF- $\kappa$ B activity as above. The results are shown in Fig. 12B. The Apo-2L induced a significant NF- $\kappa$ B activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha  
10 induced a more pronounced activation in both cell lines. Several studies have disclosed that NF- $\kappa$ B activation by TNF can protect cells against TNF-induced apoptosis [Nagata, *supra*].

The effects of a NF- $\kappa$ B inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40  $\mu$ g/ml) or cyclohexamide (Sigma) (50  $\mu$ g/ml) for 1 hour before addition of Apo-2L  
15 (1  $\mu$ g/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 12C).

The results are shown in Fig. 12C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF- $\kappa$ B-dependent genes. The data also indicates that Apo-2L is capable of activating NF- $\kappa$ B in certain cell lines and that both Apo-2 and DR4 may mediate that function.

20

#### EXAMPLE 11

##### Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase  $^{32}$ P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN  
25 (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

30 As shown in Fig. 13, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and  
35 spleen, have been shown previously to express DR4 [Pan et al., *supra*], however, the relative levels of expression of each receptor mRNA appear to be different.

### EXAMPLE 12

#### Chromosomal Localization of the Apo-2, DR4 and Apo-2DcR genes

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21. Analysis of Apo-2DcR using radiation hybrid panel examination showed that the Apo-2DcR gene is linked to the marker WI-6536, which in turn is linked to D8S298, which maps also to human chromosome 8p21 and is nested between D8S2005 and D8S2127. Thus, the human genes for three Apo-2L receptors, Apo-2, Apo-2DcR and DR4, all map to chromosome 8p21.

To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8p.

### EXAMPLE 13

#### Preparation of Monoclonal Antibodies for Apo-2DcR

Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5µg/50µl of an Apo-2DcR immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3 day intervals. The Apo-2DcR immunoadhesin protein was generated by fusing an N-terminal region of Apo-2DcR (amino acids 1-165 shown in Fig. 1A) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539(1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

Three days after the final boost, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA to test for the presence of monoclonal antibodies binding to the Apo-2DcR immunoadhesin protein.

In the ELISA, 96-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each well and incubating at 4°C overnight. The plates were then washed three times with wash buffer (PBS containing 0.05% Tween 20). The wells in the microtiter plates were then blocked with 200 µl of 2.0% bovine serum albumin in PBS and incubated at room temperature for 1 hour. The plates were then washed again three times with wash buffer.



After the washing step, 50  $\mu$ l of 0.4  $\mu$ g/ml Apo-2DcR immunoadhesin protein (as described above) in assay buffer (PBS containing 0.5% BSA) was added to each well. The plates were incubated for 1 hour at room temperature on a shaker apparatus, followed by washing three times with wash buffer.

Following the wash steps, 100  $\mu$ l of the hybridoma supernatants or purified antibody (using Protein G-sepharose columns) (1  $\mu$ g/ml) was added to designated wells in assay buffer. 100  $\mu$ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50  $\mu$ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer, was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50  $\mu$ l of substrate (TMB microwell peroxidase substrate, Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50  $\mu$ l of TMB 1-component stop solution (diethyl glycol, Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

Of the hybridoma supernatants screened in the ELISA, 47 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using HUMEK cells (a human microvascular endothelial cell line expressing Apo-2DcR; Cell Systems, Kirkland, WA) and PE-conjugated goat anti-mouse IgG. For this analysis, 25  $\mu$ l of cells suspended (at  $4 \times 10^6$  cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02%  $\text{NaN}_3$ ) were added to U-bottom microtiter wells, mixed with 100  $\mu$ l of culture supernatant or purified antibody (purified on Protein G-sepharose columns) (10  $\mu$ g/ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100  $\mu$ l PE-conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 200  $\mu$ l of cell sorter buffer and then analyzed by FACScan (Becton Dickinson, Mountain View, CA). FACS analysis showed 12/35 supernatants were positive for anti-Apo-2 antibodies.

Figure 14 shows the FACS staining of HUMEK cells incubated with the Apo-2DcR antibodies, referred to as 4G3.9.9; 6D10.9.7; and 1C5.24.1. As shown in Figure 14, the respective antibodies recognize the Apo-2DcR receptor expressed in HUMEK cells.

#### EXAMPLE 14

ELISA Assay to Test Binding of Apo-2DcR

##### Antibodies to Other Apo-2 Ligand Receptors

An ELISA was conducted to determine if the monoclonal antibodies described in Example 13 were able to bind other known Apo-2L receptors beside Apo-2DcR. Specifically, the 4G3.9.9; 6D10.9.7; and 1C5.24.1 antibodies, respectively, were tested for binding to the Apo-2DcR described herein and to DR4 [Pan et al., *supra*], Apo-2 [described in the Examples above], and DcR2 [Marsters et al., *Curr. Biol.*, 7:1003-1006 (1997)]. The ELISA was performed essentially as described in Example 13 above.

The results are shown in Figure 15. The Apo-2DcR antibody 4G3.9.9 bound to Apo-2DcR. The 4G3.9.9 antibody also showed some cross-reactivity to DR4 and Apo-2, as well as somewhat limited cross-reactivity to DcR2. The 6D10.9.7 antibody bound to Apo-2DcR and showed somewhat limited cross-

reactivity to DR4, Apo-2 and DcR2. Finally, the 1C5.24.1 antibody bound to Apo-2DcR and showed some cross-reactivity to DR4. The 1C5.24.1 antibody exhibited somewhat less cross-reactivity to Apo-2 and DcR2. A summary of the cross-reactive properties is also provided in Figure 16.

#### EXAMPLE 15

5

#### Antibody Isotyping

The isotype of the Apo-2DcR antibodies (as described above in Examples 13 and 14) was determined by coating microtiter plates with isotype specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at 4°C. The plates were then washed with wash buffer (as described in Example 13 above). The wells in the microtiter plates were then blocked with 200 µl of 2% bovine serum albumin (BSA) and incubated at room temperature for one hour. The plates were washed again three times with wash buffer. Next, 100 µl of hybridoma culture supernatant or 5 µg/ml of purified antibody was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50 µl HRP-conjugated goat anti-mouse IgG (as described above in Example 13) was added to each well. The plates were incubated for 30 minutes at room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

15 The isotyping analysis showed that the 4G3.9.9 and 1C5.24.1 antibodies are IgG1 antibodies. The analysis also showed that the 6D10.9.7 antibody is an IgG2b antibody. These results are also shown in Figure 16.

#### Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	209021	May 8, 1997
Apo2-DcR	209087	May 30, 1997
4G3.9.9	_____	_____
25 6D10.9.7	_____	_____
1C5.24.1	_____	_____

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a

license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since  
5 the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the  
10 invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.
- (ii) TITLE OF INVENTION: Apo-2DcR
- 5 (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Genentech, Inc.
- (B) STREET: 1 DNA Way
- 10 (C) CITY: South San Francisco
- (D) STATE: California
- (E) COUNTRY: USA
- (F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
- 15 (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- 20 (B) FILING DATE: 12-Jun-1998
- (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/878168
- (B) FILING DATE: 18-JUN-1997
- 25 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Marschang, Diane L.
- (B) REGISTRATION NUMBER: 35,600
- (C) REFERENCE/DOCKET NUMBER: P1110P1PCT
- (ix) TELECOMMUNICATION INFORMATION:
- 30 (A) TELEPHONE: 650/225-5416
- (B) TELEFAX: 650/952-9881
- (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 259 amino acids
- 35 (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met	Ala	Arg	Ile	Pro	Lys	Thr	Leu	Lys	Phe	Val	Val	Val	Ile	Val
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Ala	Val	Leu	Leu	Pro	Val	Leu	Ala	Tyr	Ser	Ala	Thr	Thr	Ala	Arg
40				20					25					30
Gln	Glu	Glu	Val	Pro	Gln	Gln	Thr	Val	Ala	Pro	Gln	Gln	Gln	Arg
				35					40					45

	His Ser Phe Lys Gly Glu Glu Cys Pro Ala Gly Ser His Arg Ser	50	55	60
	Glu His Thr Gly Ala Cys Asn Pro Cys Thr Glu Gly Val Asp Tyr	65	70	75
5	Thr Asn Ala Ser Asn Asn Glu Pro Ser Cys Phe Pro Cys Thr Val	80	85	90
	Cys Lys Ser Asp Gln Lys His Lys Ser Ser Cys Thr Met Thr Arg	95	100	105
10	Asp Thr Val Cys Gln Cys Lys Glu Gly Thr Phe Arg Asn Glu Asn	110	115	120
	Ser Pro Glu Met Cys Arg Lys Cys Ser Arg Cys Pro Ser Gly Glu	125	130	135
	Val Gln Val Ser Asn Cys Thr Ser Trp Asp Asp Ile Gln Cys Val	140	145	150
15	Glu Glu Phe Gly Ala Asn Ala Thr Val Glu Thr Pro Ala Ala Glu	155	160	165
	Glu Thr Met Asn Thr Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu	170	175	180
20	Glu Thr Met Asn Thr Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu	185	190	195
	Glu Thr Met Thr Thr Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu	200	205	210
	Glu Thr Met Thr Thr Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu	215	220	225
25	Glu Thr Met Thr Thr Ser Pro Gly Thr Pro Ala Ser Ser His Tyr	230	235	240
	Leu Ser Cys Thr Ile Val Gly Ile Ile Val Leu Ile Val Leu Leu	245	250	255
30	Ile Val Phe Val	259		

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1180 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGTGGGAA CCTCTCCACG CGCACGAACT CAGCCAACGA TTTCTGATAG 50

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 CGTTAGGGAA CTCTGGGGAC AGAGCGCCCC GGCCGCCTGA TGGCCGAGGC 150  
 AGGGTGCGAC CCAGGACCCA GGACGGCGTC GGAACCATA CC ATG 195  
 Met  
 5 1  
 GCC CGG ATC CCC AAG ACC CTA AAG TTC GTC GTC GTC ATC 234  
 Ala Arg Ile Pro Lys Thr Leu Lys Phe Val Val Val Ile  
 5 10  
 GTC GCG GTC CTG CTG CCA GTC CTA GCT TAC TCT GCC ACC 273  
 10 Val Ala Val Leu Leu Pro Val Leu Ala Tyr Ser Ala Thr  
 15 20 25  
 ACT GCC CGG CAG GAG GAA GTT CCC CAG CAG ACA GTG GCC 312  
 Thr Ala Arg Gln Glu Glu Val Pro Gln Gln Thr Val Ala  
 30 35 40  
 15 CCA CAG CAA CAG AGG CAC AGC TTC AAG GGG GAG GAG TGT 351  
 Pro Gln Gln Gln Arg His Ser Phe Lys Gly Glu Glu Cys  
 45 50  
 CCA GCA GGA TCT CAT AGA TCA GAA CAT ACT GGA GCC TGT 390  
 20 Pro Ala Gly Ser His Arg Ser Glu His Thr Gly Ala Cys  
 55 60 65  
 AAC CCG TGC ACA GAG GGT GTG GAT TAC ACC AAC GCT TCC 429  
 Asn Pro Cys Thr Glu Gly Val Asp Tyr Thr Asn Ala Ser  
 70 75  
 AAC AAT GAA CCT TCT TGC TTC CCA TGT ACA GTT TGT AAA 468  
 25 Asn Asn Glu Pro Ser Cys Phe Pro Cys Thr Val Cys Lys  
 80 85 90  
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 Ser Asp Gln Lys His Lys Ser Ser Cys Thr Met Thr Arg  
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 30 GAC ACA GTG TGT CAG TGT AAA GAA GGC ACC TTC CGG AAT 546  
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 GAA AAC TCC CCA GAG ATG TGC CGG AAG TGT AGC AGG TGC 585  
 35 Glu Asn Ser Pro Glu Met Cys Arg Lys Cys Ser Arg Cys  
 120 125 130  
 CCT AGT GGG GAA GTC CAA GTC AGT AAT TGT ACG TCC TGG 624  
 Pro Ser Gly Glu Val Gln Val Ser Asn Cys Thr Ser Trp  
 135 140  
 GAT GAT ATC CAG TGT GTT GAA GAA TTT GGT GCC AAT GCC 663  
 40 Asp Asp Ile Gln Cys Val Glu Glu Phe Gly Ala Asn Ala  
 145 150 155  
 ACT GTG GAA ACC CCA GCT GCT GAA GAG ACA ATG AAC ACC 702  
 Thr Val Glu Thr Pro Ala Ala Glu Glu Thr Met Asn Thr

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160              165              170
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Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu Thr Met
175              180
5 AAC ACC AGC CCA GGG ACT CCT GCC CCA GCT GCT GAA GAG 780
Asn Thr Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu
185              190              195
ACA ATG ACC ACC AGC CCG GGG ACT CCT GCC CCA GCT GCT 819
Thr Met Thr Thr Ser Pro Gly Thr Pro Ala Pro Ala Ala
10              200              205
GAA GAG ACA ATG ACC ACC AGC CCG GGG ACT CCT GCC CCA 858
Glu Glu Thr Met Thr Thr Ser Pro Gly Thr Pro Ala Pro
210              215              220
GCT GCT GAA GAG ACA ATG ACC ACC AGC CCG GGG ACT CCT 897
15 Ala Ala Glu Glu Thr Met Thr Thr Ser Pro Gly Thr Pro
225              230              235
GCC TCT TCT CAT TAC CTC TCA TGC ACC ATC GTA GGG ATC 936
Ala Ser Ser His Tyr Leu Ser Cys Thr Ile Val Gly Ile
240              245
20 ATA GTT CTA ATT GTG CTT CTG ATT GTG TTT GTT T 970
Ile Val Leu Ile Val Leu Leu Ile Val Phe Val
250              255              259
GAAAGACTTC ACTGTGGAAG AAATTCCTTC CTTACCTGAA AGGTCAGGT 1020
AGGCGCTGGC TGAGGGCGGG GGGCGCTGGA CACTCTCTGC CCTGCCTCCC 1070
25 TCTGCTGTGT TCCACAGAC AGAAACGCCT GCCCCTGCCC CAAAAAAAAA 1120
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1170
AAAAAAAAAA 1180

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## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 299 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Met Gln Gly Val Lys Glu Arg Phe Leu Pro Leu Gly Asn Ser Gly
35 -40              -35              -30
Asp Arg Ala Pro Arg Pro Pro Asp Gly Arg Gly Arg Val Arg Pro
-25              -20              -15
Arg Thr Gln Asp Gly Val Gly Asn His Thr Met Ala Arg Ile Pro
-10              -5              1              5

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	Lys Thr Leu Lys Phe Val Val Val Ile Val Ala Val Leu Leu Pro	10	15	20
	Val Leu Ala Tyr Ser Ala Thr Thr Ala Arg Gln Glu Glu Val Pro	25	30	35
5	Gln Gln Thr Val Ala Pro Gln Gln Gln Arg His Ser Phe Lys Gly	40	45	50
	Glu Glu Cys Pro Ala Gly Ser His Arg Ser Glu His Thr Gly Ala	55	60	65
10	Cys Asn Pro Cys Thr Glu Gly Val Asp Tyr Thr Asn Ala Ser Asn	70	75	80
	Asn Glu Pro Ser Cys Phe Pro Cys Thr Val Cys Lys Ser Asp Gln	85	90	95
	Lys His Lys Ser Ser Cys Thr Met Thr Arg Asp Thr Val Cys Gln	100	105	110
15	Cys Lys Glu Gly Thr Phe Arg Asn Glu Asn Ser Pro Glu Met Cys	115	120	125
	Arg Lys Cys Ser Arg Cys Pro Ser Gly Glu Val Gln Val Ser Asn	130	135	140
20	Cys Thr Ser Trp Asp Asp Ile Gln Cys Val Glu Glu Phe Gly Ala	145	150	155
	Asn Ala Thr Val Glu Thr Pro Ala Ala Glu Glu Thr Met Asn Thr	160	165	170
	Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu Thr Met Asn Thr	175	180	185
25	Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu Thr Met Thr Thr	190	195	200
	Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu Thr Met Thr Thr	205	210	215
30	Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu Thr Met Thr Thr	220	225	230
	Ser Pro Gly Thr Pro Ala Ser Ser His Tyr Leu Ser Cys Thr Ile	235	240	245
	Val Gly Ile Ile Val Leu Ile Val Leu Leu Ile Val Phe Val	250	255	259

## 35 (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1180 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

40



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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    Met Gln Gly Val Lys Glu  
 5     -40     -35

CGC TTC CTA CCG TTA GGG AAC TCT GGG GAC AGA GCG CCC 129  
 Arg Phe Leu Pro Leu Gly Asn Ser Gly Asp Arg Ala Pro  
    -30     -25

CGG CCG CCT GAT GGC CGA GGC AGG GTG CGA CCC AGG ACC 168  
 10 Arg Pro Pro Asp Gly Arg Gly Arg Val Arg Pro Arg Thr  
    -20     -15     -10

CAG GAC GGC GTC GGG AAC CAT ACC ATG GCC CGG ATC CCC 207  
 Gln Asp Gly Val Gly Asn His Thr Met Ala Arg Ile Pro  
    -5     1     5

15 AAG ACC CTA AAG TTC GTC GTC GTC ATC GTC GCG GTC CTG 246  
 Lys Thr Leu Lys Phe Val Val Val Ile Val Ala Val Leu  
    10     15

CTG CCA GTC CTA GCT TAC TCT GCC ACC ACT GCC CGG CAG 285  
 20 Leu Pro Val Leu Ala Tyr Ser Ala Thr Thr Ala Arg Gln  
    20     25     30

GAG GAA GTT CCC CAG CAG ACA GTG GCC CCA CAG CAA CAG 324  
 Glu Glu Val Pro Gln Gln Thr Val Ala Pro Gln Gln Gln  
    35     40

AGG CAC AGC TTC AAG GGG GAG GAG TGT CCA GCA GGA TCT 363  
 25 Arg His Ser Phe Lys Gly Glu Glu Cys Pro Ala Gly Ser  
    45     50     55

CAT AGA TCA GAA CAT ACT GGA GCC TGT AAC CCG TGC ACA 402  
 His Arg Ser Glu His Thr Gly Ala Cys Asn Pro Cys Thr  
    60     65     70

30 GAG GGT GTG GAT TAC ACC AAC GCT TCC AAC AAT GAA CCT 441  
 Glu Gly Val Asp Tyr Thr Asn Ala Ser Asn Asn Glu Pro  
    75     80

TCT TGC TTC CCA TGT ACA GTT TGT AAA TCA GAT CAA AAA 480  
 35 Ser Cys Phe Pro Cys Thr Val Cys Lys Ser Asp Gln Lys  
    85     90     95

CAT AAA AGT TCC TGC ACC ATG ACC AGA GAC ACA GTG TGT 519  
 His Lys Ser Ser Cys Thr Met Thr Arg Asp Thr Val Cys  
    100     105

CAG TGT AAA GAA GGC ACC TTC CGG AAT GAA AAC TCC CCA 558  
 40 Gln Cys Lys Glu Gly Thr Phe Arg Asn Glu Asn Ser Pro  
    110     115     120

GAG ATG TGC CGG AAG TGT AGC AGG TGC CCT AGT GGG GAA 597

Glu Met Cys Arg Lys Cys Ser Arg Cys Pro Ser Gly Glu  
 125 130 135  
 GTC CAA GTC AGT AAT TGT ACG TCC TGG GAT GAT ATC CAG 636  
 Val Gln Val Ser Asn Cys Thr Ser Trp Asp Asp Ile Gln  
 140 145  
 TGT GTT GAA GAA TTT GGT GCC AAT GCC ACT GTG GAA ACC 675  
 Cys Val Glu Glu Phe Gly Ala Asn Ala Thr Val Glu Thr  
 150 155 160  
 CCA GCT GCT GAA GAG ACA ATG AAC ACC AGC CCG GGG ACT 714  
 Pro Ala Ala Glu Thr Met Asn Thr Ser Pro Gly Thr  
 165 170  
 CCT GCC CCA GCT GCT GAA GAG ACA ATG AAC ACC AGC CCA 753  
 Pro Ala Pro Ala Ala Glu Glu Thr Met Asn Thr Ser Pro  
 175 180 185  
 GGG ACT CCT GCC CCA GCT GCT GAA GAG ACA ATG ACC ACC 792  
 Gly Thr Pro Ala Pro Ala Ala Glu Glu Thr Met Thr Thr  
 190 195 200  
 AGC CCG GGG ACT CCT GCC CCA GCT GCT GAA GAG ACA ATG 831  
 Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu Thr Met  
 205 210  
 ACC ACC AGC CCG GGG ACT CCT GCC CCA GCT GCT GAA GAG 870  
 Thr Thr Ser Pro Gly Thr Pro Ala Pro Ala Ala Glu Glu  
 215 220 225  
 ACA ATG ACC ACC AGC CCG GGG ACT CCT GCC TCT TCT CAT 909  
 Thr Met Thr Thr Ser Pro Gly Thr Pro Ala Ser Ser His  
 230 235  
 TAC CTC TCA TGC ACC ATC GTA GGG ATC ATA GTT CTA ATT 948  
 Tyr Leu Ser Cys Thr Ile Val Gly Ile Ile Val Leu Ile  
 240 245 250  
 GTG CTT CTG ATT GTG TTT GTT T GAAAGACTTC ACTGTGGAAG 990  
 Val Leu Leu Ile Val Phe Val  
 255 259  
 AAATTCCTTC CTTACCTGAA AGGTTTCAGGT AGGCGCTGGC TGAGGGCGGG 1040  
 GGGCGCTGGA CACTCTCTGC CCTGCCTCCC TCTGCTGTGT TCCCACAGAC 1090  
 AGAAACGCCT GCCCCTGCCC CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1140  
 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1180

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGTAAAACGA CGGCCAGTTA AATAGACCTG CAATTATTAA TCT 43

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 5 (A) LENGTH: 41 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10 CAGGAAACAG CTATGACCAC CTGCACACCT GCAAATCCAT T 41

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 49 amino acids  
 15 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Cys	Arg	Glu	Cys	Glu	Ser	Gly	Ser	Phe	Thr	Ala	Ser	Glu	Asn	His
1				5					10				15	
Leu	Arg	His	Cys	Leu	Ser	Cys	Ser	Lys	Cys	Arg	Lys	Glu	Met	Gly
20				20				25					30	
Gln	Val	Glu	Ile	Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	Cys
				35				40					45	
Gly	Cys	Arg	Lys											
				49										

25 (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 48 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Cys	Asn	Pro	Cys	Thr	Glu	Gly	Val	Asp	Tyr	Thr	Asn	Ala	Ser	Asn
1				5				10					15	
Asn	Glu	Pro	Ser	Cys	Phe	Pro	Cys	Thr	Val	Cys	Lys	Ser	Asp	Gln
				20				25					30	
Lys	His	Lys	Ser	Ser	Cys	Thr	Met	Thr	Arg	Asp	Thr	Val	Cys	Gln
				35				40					45	
Cys	Lys	Glu												

48

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 70 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50  
10 GCTAAAGCTG AGGCAGCGGG 70

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 1799 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

	CCCACGCGTC	CGCATAAATC	AGCACGCGGC	CGGAGAACCC	CGCAATCTCT	50
	GCGCCACAA	AATACACCGA	CGATGCCCGA	TCTACTTTAA	GGGCTGAAAC	100
20	CCACGGGCCT	GAGAGACTAT	AAGAGCGTTC	CCTACCGCC	ATG GAA 145 Met Glu 1	
	CAA CGG GGA	CAG AAC GCC	CCG GCC GCT	TCG GGG GCC	CGG 184	
	Gln Arg Gly	Gln Asn Ala	Pro Ala Ala	Ser Gly Ala	Arg 15	
25		5		10		
	AAA AGG CAC	GGC CCA GGA	CCC AGG GAG	GCG CGG GGA	GCC 223	
	Lys Arg His	Gly Pro Gly	Pro Arg Glu	Ala Arg Gly	Ala	
		20		25		
	AGG CCT GGG	CTC CGG GTC	CCC AAG ACC	CTT GTG CTC	GTT 262	
30	Arg Pro Gly	Leu Arg Val	Pro Lys Thr	Leu Val Leu	Val	
	30		35		40	
	GTC GCC GCG	GTC CTG CTG	TTG GTC TCA	GCT GAG TCT	GCT 301	
	Val Ala Ala	Val Leu Leu	Val Ser Ala	Glu Ser Ala		
		45		50		
	CTG ATC ACC	CAA CAA GAC	CTA GCT CCC	CAG CAG AGA	GCG 340	
35	Leu Ile Thr	Gln Gln Asp	Leu Ala Pro	Gln Gln Arg	Ala	
	55		60		65	
	GCC CCA CAA	CAA AAG AGG	TCC AGC CCC	TCA GAG GGA	TTG 379	
	Ala Pro Gln	Gln Lys Arg	Ser Ser Pro	Ser Glu Gly	Leu	

	70	75	80	
	TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT	418		
	Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp			
	85	90		
5	TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC	457		
	Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His			
	95	100	105	
	TGG AAT GAC CTC CTT TTC TGC TTG CGC TGC ACC AGG TGT	496		
10	Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys			
	110	115		
	GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC	535		
	Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr			
	120	125	130	
	AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG	574		
15	Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe Arg			
	135	140	145	
	GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA	613		
	Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr			
	150	155		
20	GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA	652		
	Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr			
	160	165	170	
	CCC TGG AGT GAC ATC GAA TGT GTC CAC AAA GAA TCA GGC	691		
25	Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly			
	175	180		
	ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT	730		
	Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu Ile			
	185	190	195	
	GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA	769		
30	Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys			
	200	205	210	
	GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT	808		
	Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly			
	215	220		
35	GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT	847		
	Gly Asp Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro			
	225	230	235	
	GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC	886		
40	Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile			
	240	245		
	TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC	925		
	Leu Gln Pro Thr Gln Val Pro Glu Gln Glu Met Glu Val			
	250	255	260	

CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964  
 Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser  
 265 270 275

5 CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003  
 Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala  
 280 285

GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042  
 Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn  
 290 295 300

10 GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081  
 Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp  
 305 310

GAC TTT GCA GAC TTG GTG CCC TTT GAC TCC TGG GAG CCG 1120  
 Asp Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro  
 15 315 320 325

CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG 1159  
 Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile Lys  
 330 335 340

20 GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198  
 Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu  
 345 350

TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237  
 Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg  
 355 360 365

25 GAT GCC TCT GTC CAC ACC CTG CTG GAT GCC TTG GAG ACG 1276  
 Asp Ala Ser Val His Thr Leu Leu Asp Ala Leu Glu Thr  
 370 375

CTG GGA GAG AGA CTT GCC AAG CAG AAG ATT GAG GAC CAC 1315  
 Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp His  
 30 380 385 390

TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA GGT AAT 1354  
 Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn  
 395 400 405

35 GCA GAC TCT GCC WTG TCC TAAGTGTG ATTCTCTTCA GGAAGTGAGA 1400  
 Ala Asp Ser Ala Xaa Ser  
 410 411

CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC 1450

AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500

CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACCTT TTCACTGCAC 1550

40 TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT 1600

GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA 1650

TTGTTTTAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT 1700

TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAG 1750

GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:11:

- 5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 411 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

10	Met	Glu	Gln	Arg	Gly	Gln	Asn	Ala	Pro	Ala	Ala	Ser	Gly	Ala	Arg	
	1				5					10					15	
	Lys	Arg	His	Gly	Pro	Gly	Pro	Arg	Glu	Ala	Arg	Gly	Ala	Arg	Pro	
					20					25					30	
	Gly	Leu	Arg	Val	Pro	Lys	Thr	Leu	Val	Leu	Val	Val	Ala	Ala	Val	
15					35					40					45	
	Leu	Leu	Leu	Val	Ser	Ala	Glu	Ser	Ala	Leu	Ile	Thr	Gln	Gln	Asp	
					50					55					60	
	Leu	Ala	Pro	Gln	Gln	Arg	Ala	Ala	Pro	Gln	Gln	Lys	Arg	Ser	Ser	
					65					70					75	
20	Pro	Ser	Glu	Gly	Leu	Cys	Pro	Pro	Gly	His	His	Ile	Ser	Glu	Asp	
					80					85					90	
	Gly	Arg	Asp	Cys	Ile	Ser	Cys	Lys	Tyr	Gly	Gln	Asp	Tyr	Ser	Thr	
					95					100					105	
	His	Trp	Asn	Asp	Leu	Leu	Phe	Cys	Leu	Arg	Cys	Thr	Arg	Cys	Asp	
25					110					115					120	
	Ser	Gly	Glu	Val	Glu	Leu	Ser	Pro	Cys	Thr	Thr	Thr	Arg	Asn	Thr	
					125					130					135	
	Val	Cys	Gln	Cys	Glu	Glu	Gly	Thr	Phe	Arg	Glu	Glu	Asp	Ser	Pro	
					140					145					150	
30	Glu	Met	Cys	Arg	Lys	Cys	Arg	Thr	Gly	Cys	Pro	Arg	Gly	Met	Val	
					155					160					165	
	Lys	Val	Gly	Asp	Cys	Thr	Pro	Trp	Ser	Asp	Ile	Glu	Cys	Val	His	
					170					175					180	
	Lys	Glu	Ser	Gly	Ile	Ile	Ile	Gly	Val	Thr	Val	Ala	Ala	Val	Val	
35					185					190					195	
	Leu	Ile	Val	Ala	Val	Phe	Val	Cys	Lys	Ser	Leu	Leu	Trp	Lys	Lys	
					200					205					210	
	Val	Leu	Pro	Tyr	Leu	Lys	Gly	Ile	Cys	Ser	Gly	Gly	Gly	Gly	Asp	

	215	220	225
	Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp		
	230	235	240
5	Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val		
	245	250	255
	Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly		
	260	265	270
	Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro		
	275	280	285
10	Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala		
	290	295	300
	Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp		
	305	310	315
15	Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg		
	320	325	330
	Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu		
	335	340	345
	Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp		
	350	355	360
20	Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp		
	365	370	375
	Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu		
	380	385	390
25	Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn		
	395	400	405
	Ala Asp Ser Ala Xaa Ser		
	410 411		

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
30. (A) LENGTH: 29 base pairs
- (B). TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

35 ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 base pairs



(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

5 AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

What is claimed is:

1. Isolated Apo-2DcR polypeptide having at least about 80% amino acid sequence identity with native sequence Apo-2DcR polypeptide comprising amino acid residues 1 to 259 of Fig. 1A (SEQ ID NO:1).
2. The Apo-2DcR polypeptide of claim 1 wherein said Apo-2DcR polypeptide has at least about 90% amino acid sequence identity.
3. The Apo-2DcR polypeptide of claim 2 wherein said Apo-2DcR polypeptide has at least about 95% amino acid sequence identity.
4. Isolated native sequence Apo-2DcR polypeptide comprising amino acid residues 1 to 259 of Fig. 1A (SEQ ID NO:1).
5. Isolated extracellular domain sequence of Apo-2DcR polypeptide comprising amino acid residues 1 to 161 of Fig. 1A (SEQ ID NO:1).
6. The extracellular domain sequence of claim 5 comprising amino acid residues 1 to 165 of Fig. 1A (SEQ ID NO:1).
7. The extracellular domain sequence of claim 5 comprising amino acid residues 1 to 236 of Fig. 1A (SEQ ID NO:1).
8. Isolated extracellular domain sequence of Apo-2DcR polypeptide comprising amino acid residues 1 to X, wherein X is any one of amino acid residues 161 to 236 of Figure 1A (SEQ ID NO:1).
9. Isolated native sequence Apo-2DcR polypeptide comprising amino acid residues -40 to 259 of Fig. 1B (SEQ ID NO:3).
10. A chimeric molecule comprising the Apo-2DcR polypeptide of claim 1 or the extracellular domain sequence of claim 5 fused to a heterologous amino acid sequence.
11. The chimeric molecule of claim 10 wherein said heterologous amino acid sequence is an epitope tag sequence.
12. The chimeric molecule of claim 10 wherein said heterologous amino acid sequence is an immunoglobulin sequence.
13. The chimeric molecule of claim 12 wherein said immunoglobulin sequence is an IgG.

14. The chimeric molecule of claim 12 wherein said extracellular domain sequence comprises amino acid residues 1 to 165 of Fig. 1A (SEQ ID NO:1).
15. An antibody which binds to the Apo-2DcR polypeptide of claim 1 or the extracellular domain sequence of claim 5.
- 5 16. The antibody of claim 15 wherein said antibody is a monoclonal antibody.
17. The antibody of claim 15 which comprises a blocking antibody.
18. The antibody of claim 15 which comprises an antibody that, in addition to binding Apo-2DcR polypeptide, binds to another Apo-2 ligand receptor.
19. The antibody of claim 15 which comprises a chimeric antibody.
- 10 20. The antibody of claim 15 which comprises a human antibody.
21. The antibody of claim 15 which comprises an IgG antibody.
22. The antibody of claim 16 having the biological characteristics of the 4G3.9.9 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_\_.
23. The antibody of claim 16 having the biological characteristics of the 6D10.9.7 monoclonal antibody  
15 produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_\_.
24. The antibody of claim 16 having the biological characteristics of the 1C5.24.1 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_\_.
25. The antibody of claim 16 wherein the antibody binds to the same epitope as the epitope to which the 4G3.9.9 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number  
20 \_\_\_\_\_ binds.
26. The antibody of claim 16 wherein the antibody binds to the same epitope as the epitope to which the 6D10.9.7 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_\_ binds.
27. The antibody of claim 16 wherein the antibody binds to the same epitope as the epitope to which the  
25 1C5.24.1 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_\_ binds.

28. A hybridoma cell line which produces the antibody of claim 16.
29. The hybridoma cell line deposited as ATCC accession number \_\_\_\_.
30. The hybridoma cell line deposited as ATCC accession number \_\_\_\_.
31. The hybridoma cell line deposited as ATCC accession number \_\_\_\_.
- 5 32. The 4G3.9.9 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_.
33. The 6D10.9.7 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_.
- 10 34. The 1C5.24.1 monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number \_\_\_\_.
35. Isolated nucleic acid comprising a nucleotide sequence encoding the Apo-2DcR polypeptide of claim 1 or the extracellular domain sequence of claim 5.
- 15 36. The nucleic acid of claim 35 wherein said nucleotide sequence encodes native sequence Apo-2DcR polypeptide comprising amino acid residues 1 to 259 of Fig. 1A (SEQ ID NO:1).
37. The nucleic acid of claim 36 wherein said nucleotide sequence comprises nucleotides 193 to 969 of Fig. 1A (SEQ ID NO:2).
38. A vector comprising the nucleic acid of claim 35.
- 20 39. The vector of claim 38 operably linked to control sequences recognized by a host cell transformed with the vector.
40. A host cell comprising the vector of claim 38.
41. The host cell of claim 40 which comprises a CHO cell.
42. The host cell of claim 40 which comprises a yeast cell.
- 25 43. The host cell of claim 40 which comprises an *E. coli*.

44. A process of using a nucleic acid molecule encoding Apo-2DcR polypeptide to effect production of Apo-2DcR polypeptide comprising culturing the host cell of claim 40.
45. A non-human, transgenic animal which contains cells that express nucleic acid encoding Apo-2DcR polypeptide.
- 5 46. The animal of claim 45 which is a mouse or rat.
47. A non-human, knockout animal which contains cells having an altered gene encoding Apo-2DcR polypeptide.
48. The animal of claim 47 which is a mouse or rat.
49. A composition comprising the Apo-2DcR of claim 1 or claim 5 and a carrier.
- 10 50. A composition comprising the Apo-2DcR antibody of claim 15 and a carrier.
51. An article of manufacture, comprising a container and a composition contained within said container, wherein the composition includes Apo-2DcR polypeptide or Apo-2DcR antibodies.
52. The article of manufacture of claim 51 further comprising instructions for using the Apo-2DcR polypeptide or Apo-2DcR antibodies *in vivo* or *ex vivo*.
- 15 53. A method of modulating apoptosis in mammalian cells comprising exposing said cells to Apo-2DcR polypeptide.
54. The method of claim 53 wherein said cells are further exposed to Apo-2 ligand.

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1 GCTGTGGGAA CCTCTCCACG CGCACGAACT CAGCCAAACGA TTTCTGATAG ATTTTGGGA GTTTGACCAG AGATGCAAGG GGTGAAGGAG CGCTTCCCTAC  
 CGACACCCCTT GGAGAGGTGC GCGTGTCTGA GTCGGTGCTT AAAGACTATC TAAAAACCCCT CAAACTGGTC TCTACGTTCC CCACTTCCTC CGAAGGATG  
 101 CGTTAGGGAA CTCTGGGGAC AGAGCGCCCC GGCCGCCCTGA TGGCCGAGGC AGGTGCGAC CCAGGACCCA GGACGGCGTC GGAACCATTA CCATGGCCCCG  
 GCAATCCCTT GAGACCCCTG TCTCGGGGG CCGGCGGACT TCCCGCTCG ACCGCGCTG GGTCCGGGT CCGTCCGCG CCGTTGGTAT GGTACCGGGC  
 1 MetalAlaArg  
 201 GATCCCCAAG ACCCTAAAGT TCGTCTGCTGT CATCGTCGG CAGTCCTAGC TTACTCTGCC ACCACTGCCC GGACGGAGGA AGTTCCCCAC  
 CTAGGGGTTT TGGGATTTC AGCAGCAGCA GTAGCAGCGC CAGGACGACG GTCAGGATCG AATGACAGCG TGGTGACGGG CCGTCTCCT TCAAGGGGTC  
 4 IleProLys ThrLeuLysP heValValVal lileValAla ValLeuLeuP roValLeuAl aTySerAla ThrThrAlaA rGlnGluGlu uValProGln  
 301 CAGACAGTGG CCCCACAGCA ACAGAGGCAC AGCTTCAAG GGGAGGAGTG TCCAGCAGGA TCTCATAGAT CAGAACATAC TGGAGCCTGT AACCCGTGCA  
 GTCTGTCACC GGGGTGCTGT TGTCTCCGTG TCGAAGTTCC CCCTCCTCAC AGGTGCTCCT AGAGTATCTA GTCTGTATG ACCTCGGACA TTGGGCAACGT  
 37 GlnThrVala laProGlnG l nGlnArgHis SerPheLysG lyGluGluCy sProAlaGly SerHisArgS erGluHisTh rGlyAlaCys AsnProCysThr  
 401 CAGAGGGTGT GGATTACACC AACGCTTCCA ACAATGAACC TTCTTGCTTC CCATGTACAG TTTCTAAATC AGATCAAAAA CATAAAAGTT CCTGCACCAT  
 GTCTCCACCA CCTAATGTGG TTGCGAAGGT TGTACTTGG AAGAACCAAG GGTACATGTC AAACATTTAG TCTAGTTTTT GTATTTTCAA GGACGTGGTA  
 71 Gluglyva laspTyThr AsnAlaSerA snAsnGluPr oSerCysPhe ProCysThrV alyCysLysSe rAspGlnLys HisLysSers erCysThrMet  
 501 GACCAGAGAC ACAGTGTGTC AGTGTAAGA AGGCACCTTC CGGAATGAAA ACTCCCCAGA GATCTGCCGG AAGTGTAGCA GTGCCCTAG TGGGGAAGTC  
 CTGGTCTCTG TGTACACACAG TCACATTTCT TCCGTGGAAG GCCTTACTTT TGAGGGGTCT CTACACGGCC TTACACATCGT CCACGGGATC ACCCTTCAG  
 104 ThrArgasp ThrValCysG l nCysLysG l uGlyThrPhe ArgAsnGluA snserProG l uMetCysArg LysCysSera rGysProse rGlyGluVal  
 601 CAAGTCAGTA ATTGTAGTGC CTGGGATGAT ATCCAGTGTG TTGAAGAATT TGGTGCCAAT GCCACTGTGG AAACCCCGAG TGTGAAGAG ACAATGAACA  
 GTTCAGTCAT TAAACATGCAG GACCCCTACTA TAGGTCACAC AACCTTCTAA ACCACGGTTA CCGTGACACC TTTGGGGTGG ACCACTTCTC TGTACTTTGT  
 137 GlnValSerA snCysThrse rTiraspasp lIeGlnCysv alGluGluPh eGlyAlaAsn AlaThrValG luThrProAl aAlaGluGlu ThrMetAsnThr  
 701 CCAGCCCCGG GACTCCTGCC CCAGCTGCTG AAGAGACAAT GAACACCAGC CCAGGGACTC CTGCCCCAGC TGCTGAAGAG ACAATGACCA CCAGCCCCGG  
 GGTCGGGGCC CTGAGGACGG GGTGACGACG TTCTCTGTTA CTGTGCTGCG GGTCCCTGAG GACGGGGTGG ACCACTTCTC TGTACTTGT GGTGGGGCCC  
 171 SerProG l yThrProAla ProAlaAlaG luGluThrMe tAsnThrSer ProGlyThrP roAlaProAl aAlaGluGlu ThrMetThrT hrSerProGly

FIG. 1A-1

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801 GACTCCTGCC CCAGCTGCTG AAGAGACAAT GACCACCAGC CCGGGGACTC CTGCCCCAGC TGCTGAAGAG ACAATGACCA CCAGCCCCGG GACTCCTGCC  
 CTGAGGACGG GGTCGACGAC TTCTCTGTTA CTGGTGGTCG GGGCCCTGAG GACGGGGTCG AGCACTTCTC TGTTACTGGT GGTGGGGCCC CTGAGGACGG  
 204 ThrProAla ProAlaAlaG luGluThrMe tThrThrser ProGlyThrP roAlaProAl aAlaGluGlu ThrMetThrT hrSerProGl yThrProAla  
 901 TCTTCTCATT ACCTCTCATG CACCATCGTA GGGATCATAG TTCTAATTGT GCTTCTGATT GTGTTTGTTC GAAGACTTC ACTGTGGAAG AAATTCCTTC  
 AGAAGAGTAA TGGAGAGTAC GTGGTAGCAT CCCTAGTATC CCCTAGTATC CGAAGACTAA CACAAACAAA CTTTCTGAAG TGACACCTTC TTTAAGGAAG  
 237 SerSerHist yrLeuSerCy sThrIleVal GlyIleIleVal lleuIleuValPheVal  
 1001 CTTACCTGAA AGGTTACGGT AGGCGCTGGC TGAGGGGGGG GGGCGCTGGA CACTCTCTGC CCTGCCCTCC TCTGCTGTGT TCCACACAGC AGAAACGGCT  
 GAATGGACTT TCCAGTCCA TCCGGGACCG ACTCCGGCCC CCGCGGACCT GTGAGAGACG GGACGGAGGG AGACGACACA AGGTGTCTG TCTTGGCGGA  
 1101 GCCCTGCC CAAAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA  
 CCGGACCGG GTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT

**FIG. 1A-2**

1 GCTGTGGGAA CCTCTCCAG CGCAGGAACT CAGCCACCA TTTCTGATAG ATTTTGGGA GTTTGACCAG AGATGCAAGG GGTGAGGAG CGCTTCCTAC  
 CGACACCCCTT GGAGAGGTGC GCGTGCTTGA GTCGGTGGCT TAAACCCCT CAAACTGGTC TCTACGTTCC CCACCTCCTC CGGAAGGATG  
 MetGlnG l yValLysGlu ArgPheLeuPro

-40

101 CGTTAGGGAA CTCTGGGGAC AGAGCGCCCC GCGCGCTCA TGGCCGAGGC AGGTGCGGAC CCAGGACCCA GGACGGCGTC GGGAAACATA CCATGGCCCC  
 GCAATCCCTT GAGACCCCTG TCTCGCGGGG CCGCGGACT ACCGCTCCG TCCACGCTG GGTCTGGGT CCTGCGCAG CCTTGGTAT GGTACCGGGC  
 -30 LeuGlyAs nSerGlyAsp ArgAlaProA rgProProAs pGlyArgGly ArgValArgP roArgThrGl nAspGlyVal GlyAsnHist hrMetAlaArg  
 201 GATCCCAAG ACCCTAAAGT TCGTGTGCTGT CATGTGCTGG GTCTGTGCTG CAGTCTGTGC TTACTCTGCC ACCACTGCCC GGCAGGAGGA AGTTCCCCAG  
 CTAGGGGTC TGGGATTTC ACGACGAGCA GTAGCAGCG CAGGACGACG GTCAGGATCG AATGAGACGG TGGTACGGG CCGTCTCTCT TCAAGGGGTC  
 4 IleProLys ThrLeuLysP heValValVal lIleValAla ValLeuLeup rovalleuAl aTySerAla ThrThrAlaA rgGlnGluG l uValProGln  
 301 CAGACAGTGG CCCACAGCA ACAGAGGCAC AGCTTCRAGG GGGAGGAGTG TCCAGCAGGA TCTCATAGAT CAGAACATAC TGGAGCCTCT AACCCGTGCA  
 GTCTGTCAAC GGGGTGCTGT TGTCTCCGTG TCGAAGTTCC CCCTCTCTAC AGTCTCTCT AGAGTATCTA GTCTGTATG ACCTCGGACA TTGGGCACGT  
 37 GlnThrVala laProGlnG l nGlnArgHis SerPheLysG lyGluGluCy sProAlaGly SerHisArg sArgGlnHisth rGlyAlaCys AsnProCysThr

**FIG. 1B-1**

401 CAGAGGGTGT GGATTACACC AACGCTTCCA ACAATGAACC TTCTTGCTTC CCATGTACAG TTTGTAATC AGATCAAAAA CATAAAAAGTT CCTGCACCAT  
 GTCTCCACA CCTAATGTGG TTGCGAAGGT TGTACTTGG AAGAACGAAG GGTACATGTC AAACATTTAG TCTAGTTTTT GTATTTTCAA GGACGTGGTA  
 71 GluGlyVa LaspyrThr AsnAlaSerA snAsnGluPr oSerCysPhe ProCysThrV alCysLysSe rAspGlnLys HisLysSers erCysThrMet  
 501 GACCAGAGAC ACAGTGTGTC AGTGTAAGA AGGCACCTTC CGGAATGAAA ACTCCCCAGA GATGTGCCGG AAGTGTAGCA GTGCCCCTAG TGGGGAAGTC  
 CTGGTCTCTG TGTACACAGC TCACATTCTT TCCGTGGAAG GCCTTACTTT TGAGGGGTCT CTACACGGCC TTCACATCGT CCACGGGATC ACCCCTTCAG  
 104 ThrArgAsp ThrValCysG lncysLysG1 uGlyThrPhe ArgAsnGluA snSerProG1 uMetCysArg LysCysSerA rgCysProse rGlyGluVal  
 601 CAAGTCAGTA ATTGTACGTC CTGGGATGAT ATCCAGTGTG TTGAAGAATT TGGTGCCAAT GCCACTGTGG AAACCCCGAG TCGTGAAGAG ACAATGAACA  
 GTTCAGTCAT TAACATGCAG GACCTACTA TAGGTCACAC AACTTCTTAA ACCACGGTTA CGGTGACACC TTTGGGGTCG ACGACTTCTC TGTACTTGT  
 137 GlnValSerA snCysThrse rTrpAspAsp IleGlnCysV alGluGluPh eGlyAlaAsn AlaThrValG luThrProAl aAlaGluGlu ThrMetAsnThr  
 701 CCAGCCCCGG GACTCCTGCC CCAGCTGCTG AAGAGACAAAT GAACACCAGC CCAGGGACTC CTGCCCCAGC TGCTGAAGAG ACAATGACCA CCAGCCCCGG  
 GGTGGGGCCC CTGAGGACGG GGTGACGAC TTCTCTGTTA CTGTGGTTCG GGTCCCTGAG GACGGGGTCG ACGACTTCTC TGTACTTGT GGTGGGGCCC  
 171 SerProG1 yThrProAla ProAlaAlaG luGluThrMe tAsnThrSer ProGlyThrP roAlaProAl aAlaGluGlu ThrMetThrT hrSerProGly  
 801 GACTCCTGCC CCAGCTGCTG AAGAGACAAAT GACCACCAGC CCAGGGACTC CTGCCCCAGC TGCTGAAGAG ACAATGACCA CCAGCCCCGG GACTCCTGCC  
 CTGAGGACGG GGTGACGAC TTCTCTGTTA CTGTGGTTCG GGTCCCTGAG GACGGGGTCG ACGACTTCTC TGTACTTGT GGTGGGGCCC CTGAGGACGG  
 204 ThrProAla ProAlaAlaG luGluThrMe tThrThrSer ProGlyThrP roAlaProAl aAlaGluGlu ThrMetThrT hrSerProG1 yThrProAla  
 901 TCTTCTCATT ACCTCTCATG CACCATCGTA GGGATCATAG TTCTAATTGT GCTTCTGATT GTGTTTCTTTT GAAAGACTTC ACTGTGGAAG AAATTCCTTC  
 AGAAGAGTAA TGGAGAGTAC GTGGTAGCAT CCTAGTATC AAGATTAAAC CGAAGACTAA CACAAACAAA CTTTCTGAAG TGACACCTTC TTTAAGGAAG  
 237 SerSerHist yrLeuSerCy sThrIleVal GlyIleIleV alLeuLeuIle ValPheVal  
 1001 CTTACCTGAA AGGTTACAGT AGGCGCTGGC TGAGGGCGGG GGGCGCTGGA CACTCTCTGC CCTGCCCTCC TCTGCTGTGT TCCCACAGAC AGAAACGCCT  
 GAATGGACTT TCCAAGTCCA TCCGGACCG ACTCCGCCC CCGCGACCT GTGAGAGACG GGACGGAGGG AGACGACACA AGGGTGTCTG TCTTGGGA  
 1101 GCCCTGCCC CAAAAAAA CAAAAAAA CAAAAAAA CAAAAAAA CAAAAAAA CAAAAAAA CAAAAAAA CAAAAAAA CAAAAAAA CAAAAAAA  
 CCGGACGGG GTTTTTTTT TTTTTTTT TTTTTTTT TTTTTTTT TTTTTTTT TTTTTTTT TTTTTTTT TTTTTTTT TTTTTTTT TTTTTTTT

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FIG. 1B-2



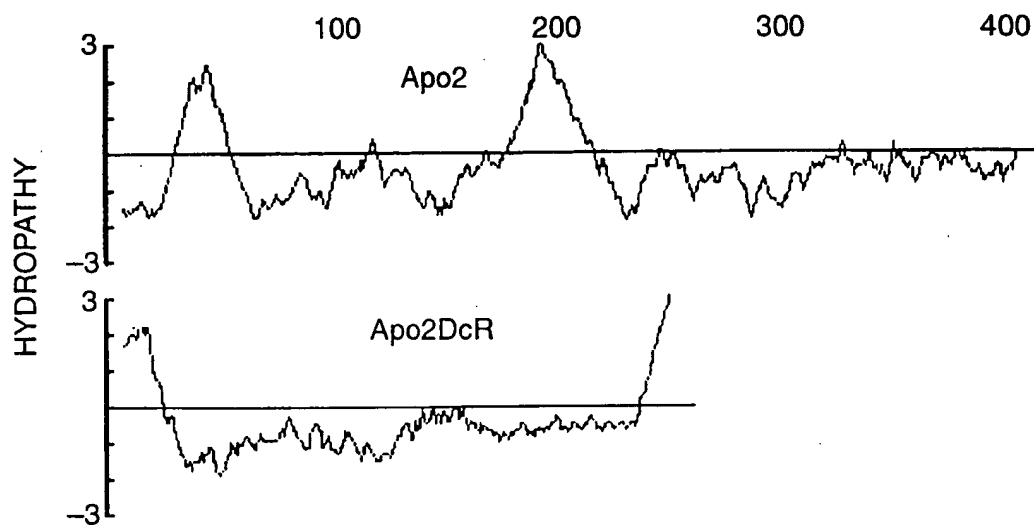
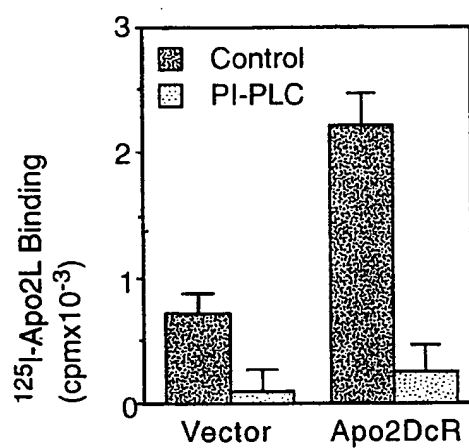
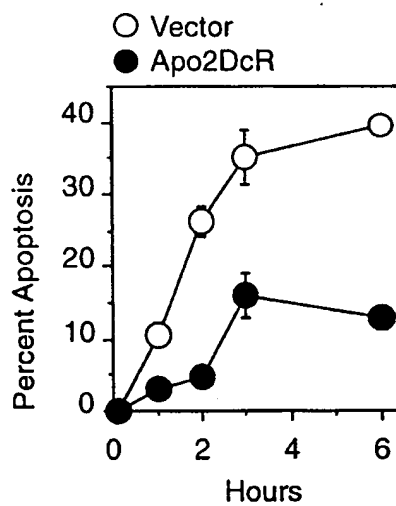
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Apo2	1	-----MEQRGQNAFAASGARKRHGPGPREARGARPGLRVPKTI
Apo2DcR	1	-----MARIPKTLKFV
DR4	51	GRGALPTSMGQHGPSARARAGRAPGERPAREASPRLRVHKTFFKVVVGVL
Apo2	41	VVAVLLLVSAESALITQDLAPQORAAPQOKRSSPSEGLCPFGHHISED
Apo2DcR	13	VIVAVLLFVLAYSATTARQEEVPQOTVAPQQRHSFKGEECPAGSHRSEH
DR4	101	LQVVPSSAATIK-----LHDSIGTQQWEHSPLGELCPFGSHRSEH
Apo2	91	GRDCISCKYGGDYSTHWNDLLFCLRCTRCDSGEVELSPCTTTRNTVCQCE
Apo2DcR	63	TGACNECTEGVDYTNASNNEPSCFPCTVCKSDQKHSSCTMTRDITVCQCK
DR4	142	FGACNRCTEGVGYTNASNLLFACLPCTACKSDEEERSPCTTTRNTACQCK
Apo2	141	EGTFREEDSPERMCRKCHTGCPRGMVKVGDCPTWSDIECVHKE-----
Apo2DcR	113	EGTFRNENSPERMCRKCSR-CPSGEVQVSNCTSWDDIQCVE-EFGANATVE
DR4	192	EGTFRNDNSAEMCRKCSGCPRGMVKVKDCTPWSIECVHKE-----
Apo2		-----
Apo2DcR	161	TPAAETMNTSPGTPAPAAETMNTSPGTPAPAAETMTTSPGTPAPAAE
DR4		-----
Apo2	183	-----SGIILIGVTVAAVVLIVAVEV-----
Apo2DcR	211	ETMTTSPGTPAPAAETMTTSEGTAPASHYLSCTIVGIIVLIVLLIVFV
DR4	234	-----SNGHNIWVILVVTLLVPLLVAV-LIVC
Apo2	203	CKSLWLKVKVLPYLKGICSGGGGDPFRVDRSSQRPGAEDNVLNEIVSILQP
DR4	262	CCIGSGCGGDEKCMDRVCFWRLGLIRGPGAEDNAHNEILSNADSLSTFVS
Apo2	253	TQVPEQEMEVOEFAEFTGVNMLSPGESEHLLPAAEERSQRRRLVLPANE
DR4	312	---EQMESQEPADLTGVTVQSPGEAQCLLPAAEAGSQRRRLVLPANG
Apo2	303	GDPTEITLRQCFDDFADLVFEDSWEPLMRKLGIMDNEIKVARAEAAAGH--R
DR4	358	ADPTETIMLFEDKFNIVPFDSDWQLMRQLDLTKNEIDVVRAGTAGP--G
Apo3/DR3	338	VMDAVPARRWKEFVRTLGIREAEIEAVEVEI-GRF-R
TNFR1	322	VVENVPPLRWKEFVRRGLSDHEIDRIELON-GRCLR
CD95	220	IAGVHTLSQVKGFRKNGVNEAKIDEIKNDN-VQDTA
Apo2	351	DTLYTMIWKVNKTGR-DASVHTLLDALETIGERIAKOKIEDHLLSSGKF
DR4	406	DALYAMIMKVNKTGR-NASVHTLLDALERMERHAKKIKDILLVDSGKF
Apo3/DR3	374	DOQYEMIKRWROQQP---AGLGAVYAALERMGLDGCVEDLRS
TNFR1	358	EAQYSMLATWRRRTERRREATLELLGRVLRDMOLLGCLEDIEE
CD95	256	EOKVQLLRNWHQLHGKKEAY-DTLIKDLKKANLCTLAERLQT
Apo2	400	MYLEGNAISALS
DR4	455	IYLEDGTGSAVSLE

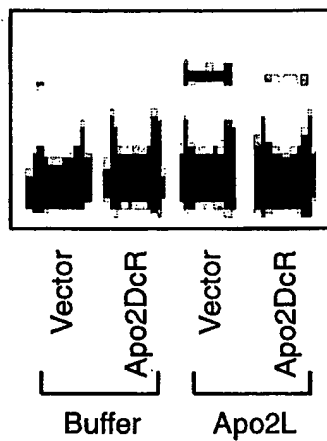
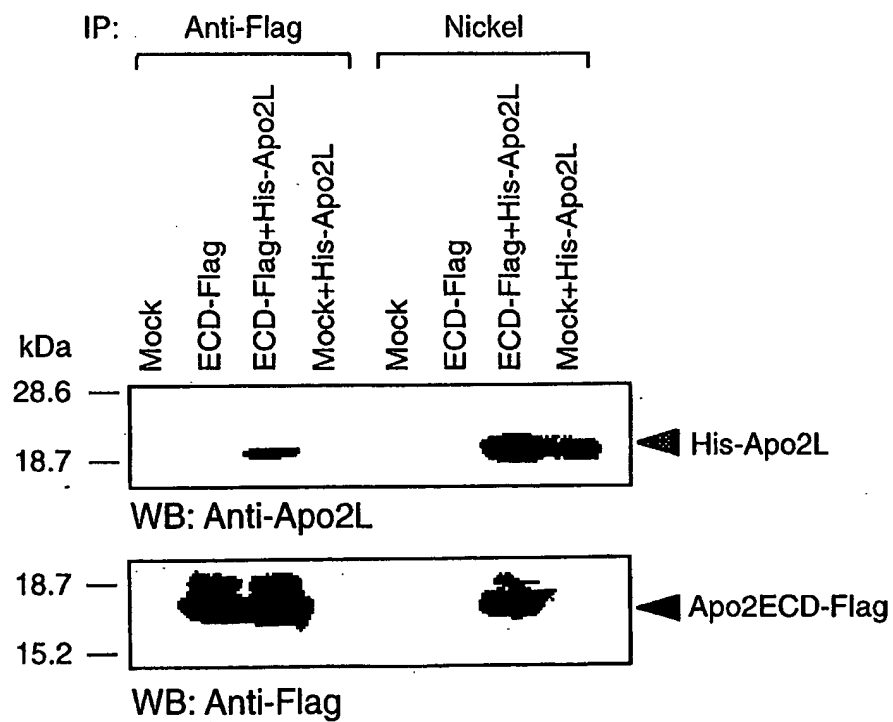
FIG. 2

SUBSTITUTE SHEET (RULE 26)

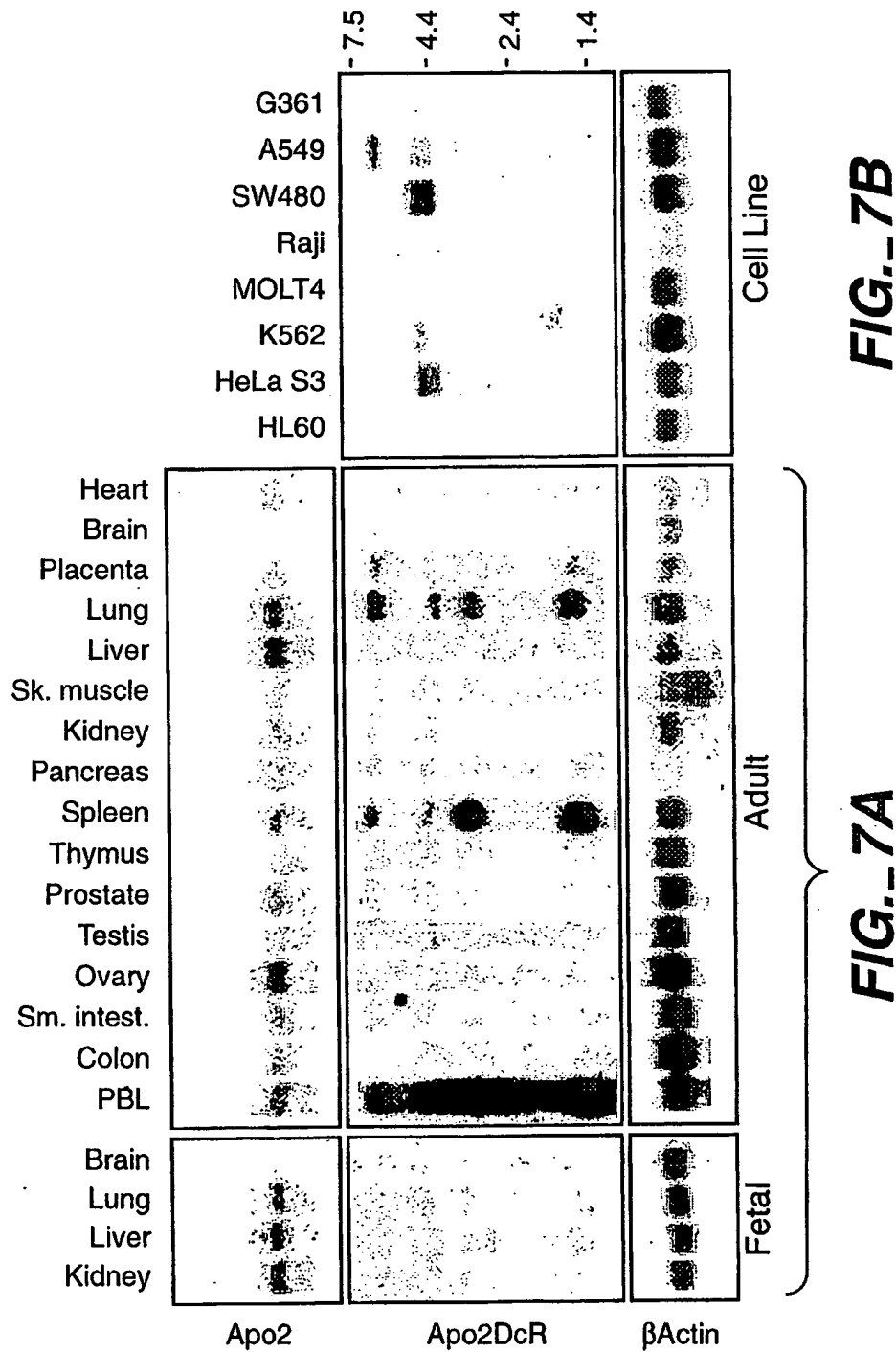
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**FIG. 3****FIG. 4****FIG. 5**

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**FIG.\_6****FIG.\_10**

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1 CCCACGGCTC CGCATAAATC AGCAGCGGGC CGGAGAACCC CGCCCCACAA AATACACCCA CGATGCCCGA TCTACTTTAA GGGCTGAAC  
 GGGTGGCCAG GCGTATTAG TCGTGGCCG GCTCTTGG GCGGTAGAG GCGGGTCTT TTATGTGGCT GCTACGGGCT AGATGAAATT CCCGACTTTC  
 101 CCACGGGCTT GAGAGACTAT AAGAGCGTTC CCTACCGCCA TGGACAACG GGCACAGAAC GCGCGGGCG CTTGCGGGG CCGGAAAAGG CACGGGCCAG  
 GGTGCCCGGA CTCTCTGATA TTCTCGCAAG GGATGGCGGT ACCTGTGTC CCCTGTCTTG CCGGGCGGG GAAGCCCCCG GGCCTTTTC GTGCCGGGTC  
 1 M etGluGlnAr gGlyGlnAsn AlaProAlaA laSerGlyAl aArgLysArg HisGlyProGly  
 201 GACCCAGGGA GCGCGGGGA GCGAGGCGTG GCTCCGGGT CCCAAGACC CTTGTGCTCG TTGTGCGCGG GTCTCTGCTG TTGCTCTCAG CTGAGTCTGC  
 CTGGTCCCT CCGCGCCCT CCGTCCGGAC CCGAGGCCCA GGGTCTCTGG GAACACGAGC AACAGCGGCG CCAGGACGAC AACACAGATC GACTCAGACG  
 22 ProArgGl uAlaArgGly AlaArgProG lyLeuArgVa lProLysThr LeuValLeuV alValAlaAl aValLeuLeu LeuValSerA laGluSerAla  
 301 TCTGATCACC CAACAAGACC TAGCTCCCA TAGCTCCCA GCAGAGAGCG GCCCACAAAC AAAAGAGGTC CAGCCCTCA GAGGATTGT GTCCACCTGG ACACCATATC  
 AGACTAGTGG GTTGTCTTGG ATCAGGGGT ATCAGGGGT CCGTCTCTCG CCGGTCTTG TTTTCTCCAG GTCCGAGAT CTCCCTAACA CAGGTGGACC TGTGGTATAG  
 55 LeuileThr GlnGlnAspL euAlaProGl nGlnArgAla AlaProGlnG lNlysArgse rserProSer GluglyLeuc ysProProGl yHisHisile  
 401 TCAGAAAGCG GTAGAGATTG CATCTCTGCG AATATGGAC AGGACTATAG CACTCACTGG AATGACTCC TTTTCTGCTT GCCTGCACC AGGTGTGATT  
 AGTCTCTGC CATCTCTAAC GTAGAGGACG TTTATACCTG TCTGATATC GTGAGTGACC TTACTGGAGG AAAAGACGAA CCGGACGTGG TCCACACTAA  
 88 SerGluaspG lyArgAspCy sIleSerCys LysTyrGlyG lNAspTyrSe rThrHisTirp AsnAspLeuL eupheCysLe uArgCysThr ArgCysaspSer  
 501 CAGGTGAAGT GGAGCTAAGT CCGTGCACCA CGACCAGAAA CACAGTGTGT CAGTGCGAAG AAGGCACCTT CCGGGAAGAA GATTCTCCTG AGATGTGCCG  
 GTCCACTTCA CCTCGATTCA GGGACCTGGT GCTGGTCTTT GTGTACACA GTACACGCTT TCCGTGGA GGCCTTCTT CTAAGAGGAC TCTACACGGC  
 122 GlyGluVa lGluLeuSer ProCysThrT hrThrArgAs nThrValCys GlnCysGluG luGlyThrPh eArgGluGlu AspSerProG luMetCysArg  
 601 GAAGTCCCGC ACAGGGTGTG CCACAGGGAT GGTCAGGTC GGTCATTGTA CACCTGGAG TGACATCGAA TGTGTCCACA AAGAATCAGG CATCATCATA  
 CTTACCGCGG TGTCCACAG GGTCTCCCTA CCAGTTCAG CCACATAACAT GTGGGACCTC ACTGTAGCTT ACACAGGTGT TTCTTAGTCC GTAGTAGTAT  
 155 LysCysArg ThrGlyCysP roArgGlyMe tValLysVal GlyAspCysT hrProTirpse rAspIleGlu CysValHisL ysGluSerGl yIleIleIle  
 701 GGAGTCACAG TTGCAGCGGT AGTCTTGATT GTGGCTGTGT TTGTTTGCAA GTCTTTACTG TGAAGAAAG TCCTTCTCTTA CCGGAAAGGC ATCTGCTCAG  
 CCTCAGTGTG AACGTGGCA TCAGAACTAA CACCGACACA AACAAACGTT CAGAAATGAC ACCTTCTTTC AGGAAGGAAT GGACTTTCCG TAGACGAGTC  
 188 GlyValThrV alAlaAlaVa lValleuile ValAlaValP heValCysLy sserLeuLeu TriPlysLysV alLeuProty rleuLysGly ileCysSerGly

FIG.-8A-1

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801 GTGGTGGTGG GGACCCCTGAG CGTCTGGACA GAAGCTCACA ACGACCTGGG GCTGAGGACA ATGTCTCTCAA TGAGATCGTG AGTATCTTGC AGCCCAACCCA  
 CACCACCACC CCTGGGACTC GCACACCTGT CTTCGAGTGT TGCTGGACCC CGACTCCTGT TACAGGAGTT ACTCTAGCAC TCATAGAACG TCGGGTGGGT  
 222 GlyGlyG1 yAspProGlu ArgValAspA rGserSerG1 nArgProGly AlaGluAspA snValLeuAs nGluIleVal SerIleLeuG InProThrGln  
 901 GGTCCTCTGAG CAGGAATGG AAGTCCAGGA GCCAGCAGAG CCAACAGGTG TCAACATGTT GTCCCCCGGG GAGTCAGAGC ATCTGCTGGA ACCGGCAGAA  
 CCAGGGACTC GTCCTTTACC TTCAGGTCCT CCGTCGTCTC GGTGTCCAC AGTTGTACAA CAGGGGGCCC CTCAGTCTCG TAGACGACCT TGGCCGTCTT  
 255 ValProGlu GlnGluMetG luValGlnG1 uProAlaGlu ProThrGlyV alasnMetLe uSerProGly GluSerGluH isLeuLeuG1 uProAlaGlu  
 1001 GCTGAAGAAGT CTCAGAGGAG GAGGCTGCTG GTTCCAGCAA ATGAAGGTGA TCCCACCTGAG ACTCTGAGAC AGTGCTTCGA TGACTTTTGA GACTTGGTGC  
 CGACTTTCCA GAGTCTCCTC CTCGAGGAC CAAGGTCGTT TACTTCCACT AGGGTGACTC TGAGACTCTG TCACGAAGCT ACTGAAACGT CTGAACCCACG  
 288 AlaGluArgS erGlnArgAr gArgLeuLeu ValProAlaa snGluGlyAs pProThrGlu ThrLeuArgG InCysPheAs pAspPheAla AspLeuValPro

**FIG.-8A-2**

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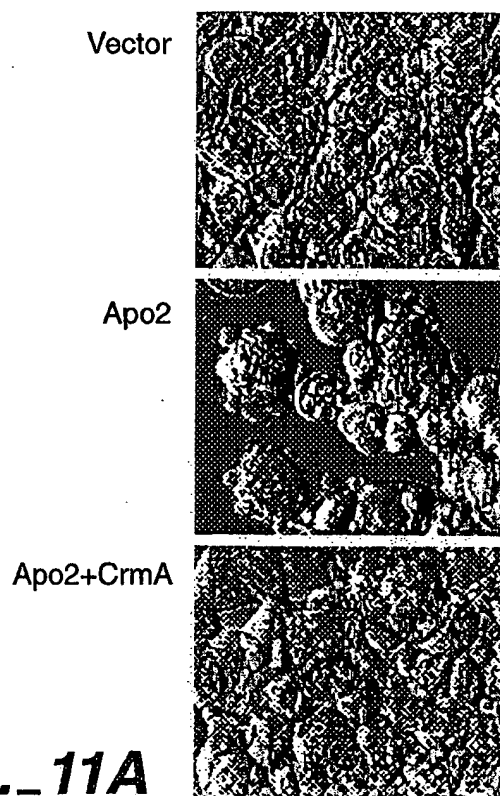
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 GGAACCTGAG GACCTCGGC GAGTACTCCT TCAACCCGGA GTACCTGTTA CTCTATTCC ACCGATTTCC ACTCCGTCC CCGGTGTCCC TGTGGRACAT  
 322 PheAspse rTrpGluPro LeuMetArgL ysLeuGlyLe ysLeuGlyLe uMetAspAsn GluileLysV alAlaLysAl aGluAlaAla GlyHisArgA spThrLeuTyf  
 1201 CACGATGCTG ATAAAGTGGG TCAACAAAAC CGGGCAGAT GCCTCTGTCC ACACCTCTCT GGATGCCTTG GAGACGCTGG GAGAGAGACT TGCCAAAGCAG  
 GTGCTACGAC TATTTCACCC AGTTGTTTTG GCCCGCTCTA CGGAGACAGG TGTGGGACGA CCTACGGAAAC CTCTCGGACC CTCTCTCTGA ACGGTTCTGC  
 355 ThrMetLeu IleLysTrpV alAsnLysTh rGlyArgAsp AlaservAlH isThrLeuLe uAspAlaLeu GluThrLeuG lyGluArgLe uAlaLysGln  
 1301 AAGATTGAGG ACCACTTGTT GAGCTCTGGA AAGTTTCATGT ATCTAGAAGG TAATGCAGAC TCTGCCWTGT CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA  
 TTCTAACTCC TGTGAAACAA CTCGAGACCT TTCAAGTACA TAGATCTTCC ATTACGCTCG AGACGGAAAC GGATTTCACAC TAAGAGAAGT CCTTCACCTCT  
 388 LysIleGluA spHisLeuLe uSerSerGly LysPheMetT yrLeuGluGl yAsnAlaAsp SerAlaXqS erOC\*  
 1401 CCTTCCCTGG TTACCTTTT TTCTGGAAA AGCCCAACTG GACTCCAGTC AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC  
 GGAAGGGACC AAATGGAAA AGACCTTTT TCGGGTTGAC CTGAGGTCAG TCATCCTTTC ACGGTGTTAA CAGTGACTCG GCCATGACCT TCTTTGAGAG  
 1501 CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACCTGCAC TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT  
 GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTTC ACTTACACTA TTATTCTCTGT GATACCTTTA  
 1601 GTCTGGATCA TTCCGTTGT GCGTACTTTG AGATTGCTT TGGGATGTC TTTGTTTTCAC AGCACTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT  
 CAGACCTAGT AAGGCAACA CGCATGAAC TCTRAACCA ACCCTACAGT AACRAAAGTG TCGTGAANA ATAGGATTAC ATTTACGAAA TAAATAAATA  
 1701 TTGGGCTACA TTGTAAGATC CATCTACAA AAAAAAAA GCGCGCCGG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC  
 AACCCGATGT AACATTCTAG GTAGATGTT TTTTTTTTT TTTTTTTT CCGCGCGGC TGAGATCTCA GCTGGACGTC TTCGAACCGG CCGTACCGG

FIG.-8B

1 MEQRGQNPAAASGARKRHGPGPREARGARPLRVPKTLVLVVAVLLLVSAESALITQOD  
 61 LAPQORAAPQOKRSSPSEGLCPPGHHISEDGRDCISCKYQDYSTHWNDDLFLCLRCTRCD  
 121 SGEVELSPCTTTRNTVCQCEGTFREEDSPEMCRKRTGCPRGVMKVGDCPTPWSDIÉCVH  
 181 KESGIIIGVTVAAVLIVAFVCKSLMKKVL PYLKGICSGGGDPERVDRSSORPGEAD  
 241 NVLNEIVSILQPTQVPEQEMEVQEPAPTGVNMLSPGESEHLLLEPAEAERSQRRLLVPA  
 301 NEGDPTELTROCFDDFADLVFFDSWEPLMRKILGLMDNEIKVAKAEAGHRDTLYTMLIKW  
 361 VNKTGRDASVHTLLDLETLGERLAKQKIEDHLLSSGKFMYLEGNADSALS

FIG.-9

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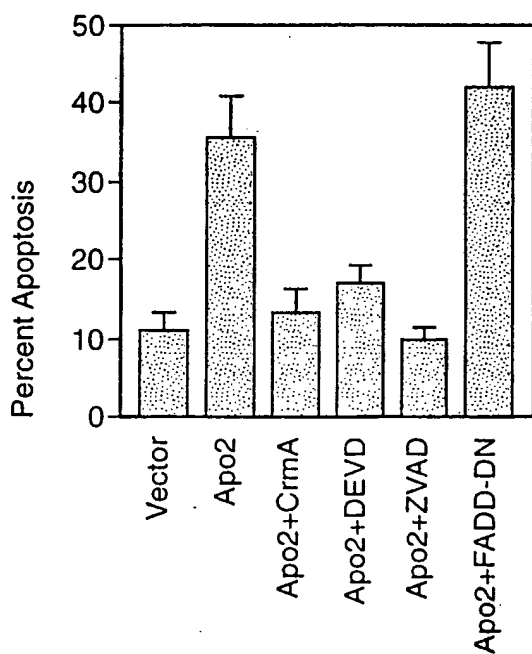
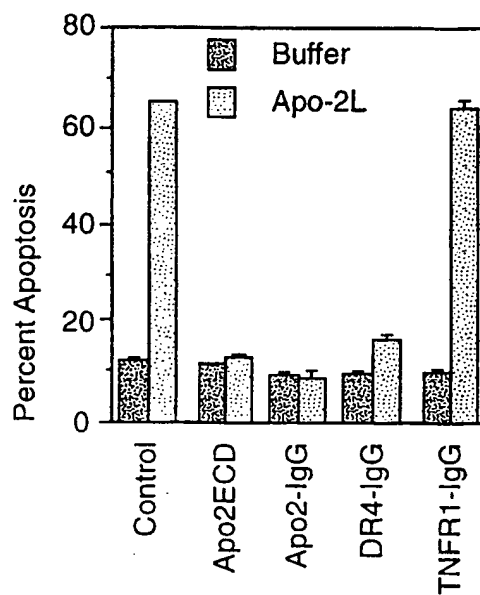
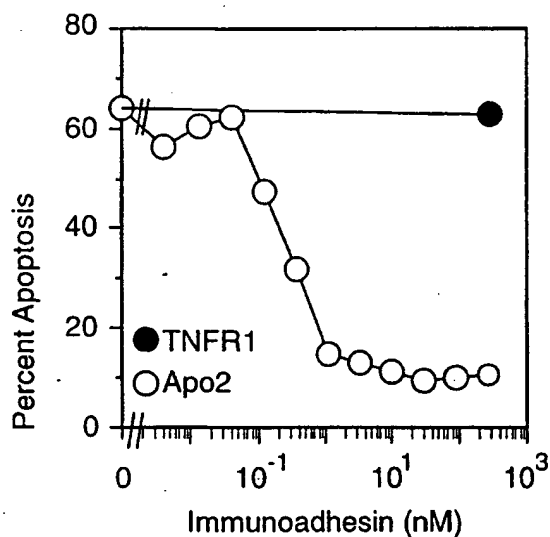
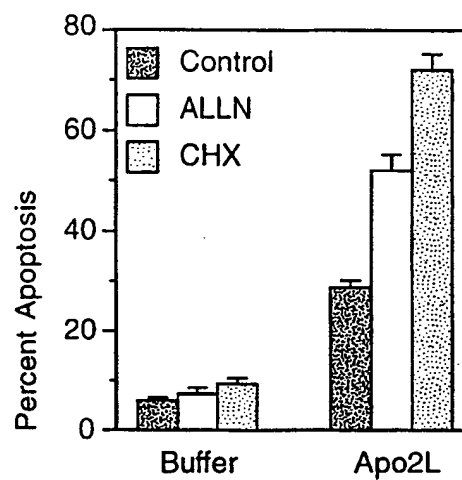
**FIG. 11A**

Vector  
Apo2  
Apo2+CrmA  
Apo2+DEVD  
Apo2+ZVAD

**FIG. 11B**

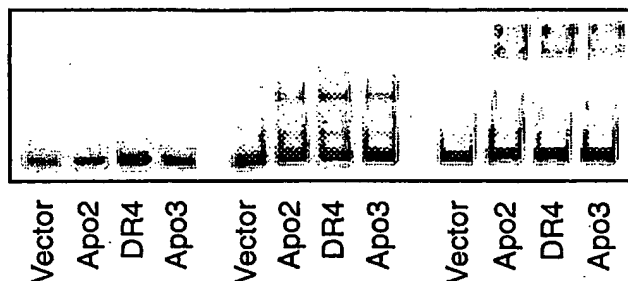


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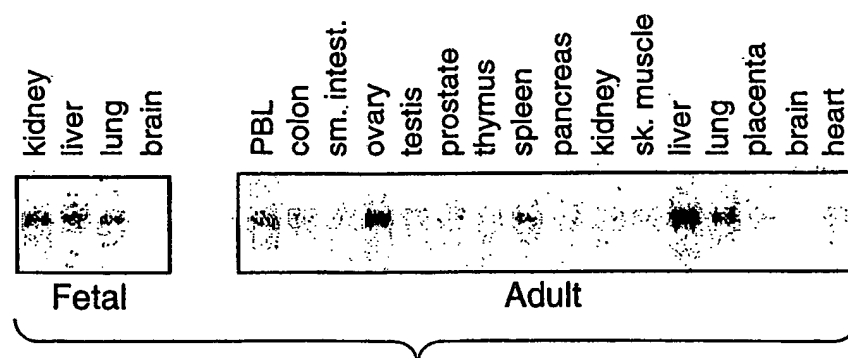
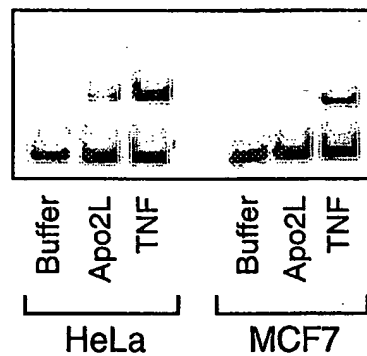
**FIG. 11C****FIG. 11D****FIG. 11E****FIG. 12C**

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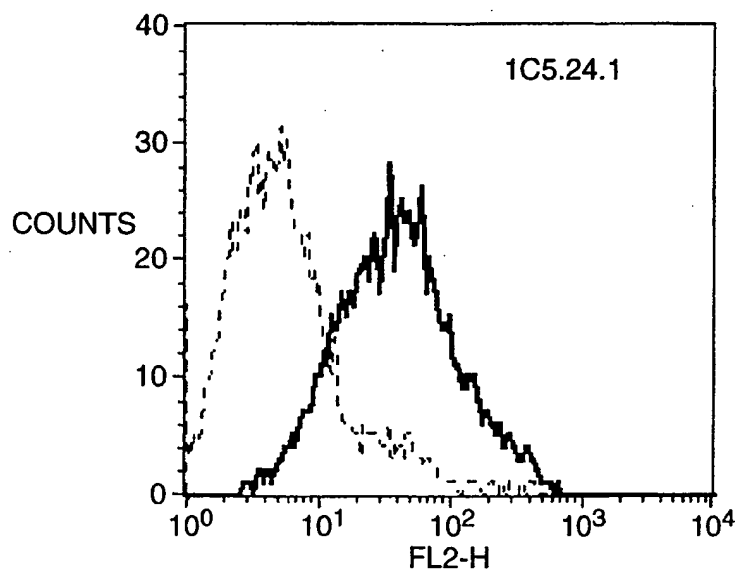
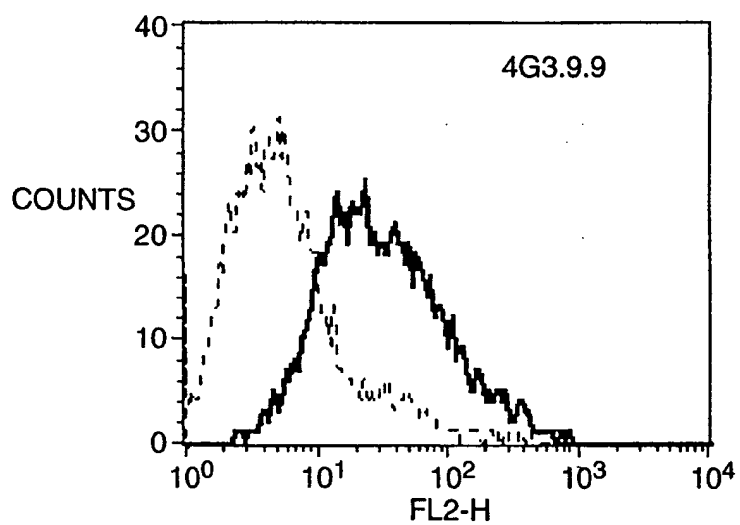
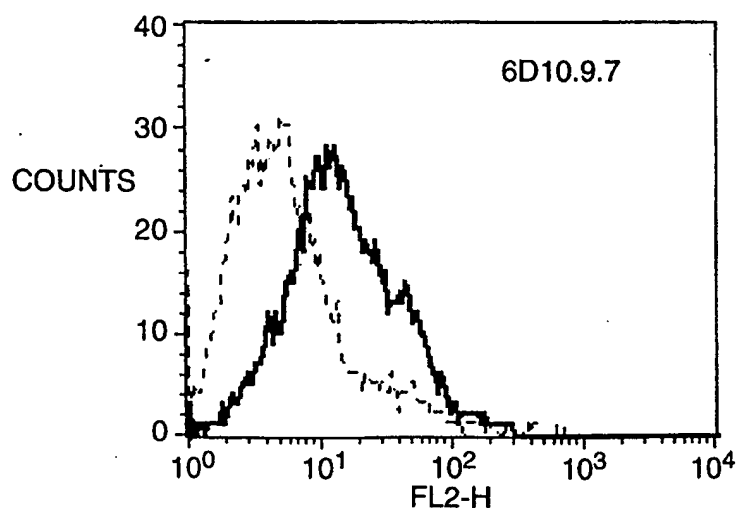
Unlabelled probe	+	+	+	+	-	-	-	-	-	-	-	-
Labelled probe	+	+	+	+	+	+	+	+	+	+	+	+
Anti-p65	-	-	-	-	-	-	-	-	+	+	+	+

**FIG. 12A**

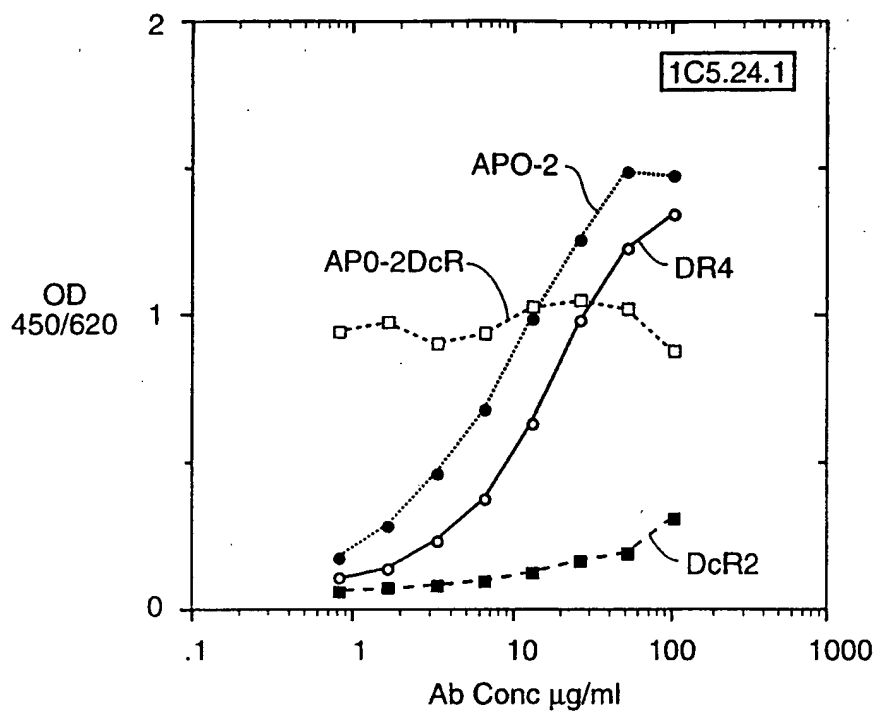
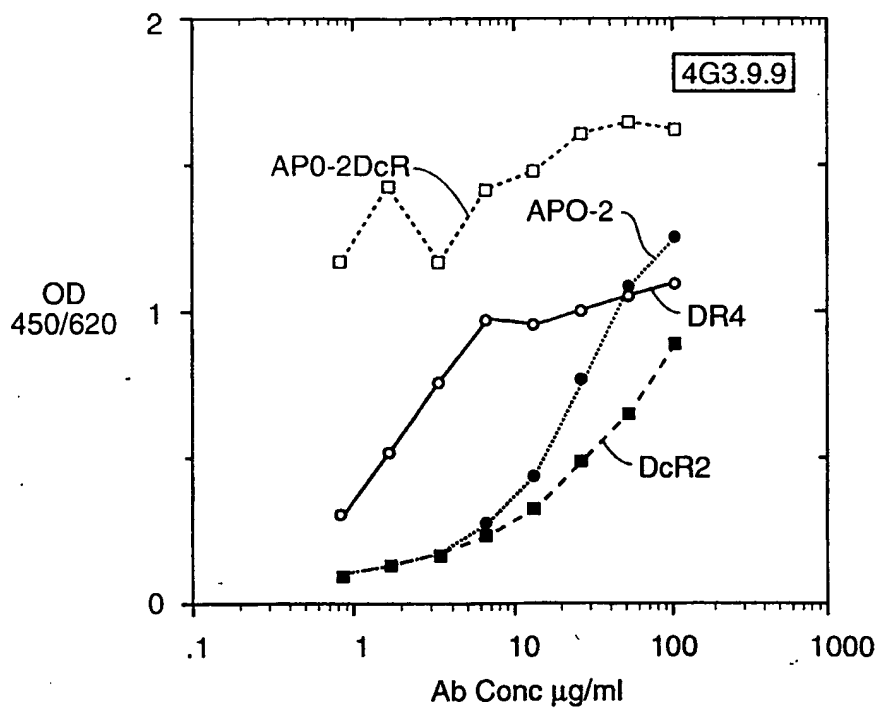
Unlabelled probe	-	-	-	-	-	-
Labelled probe	+	+	+	+	+	+
Anti-p65	-	-	-	-	-	-

**FIG. 12B****FIG. 13**

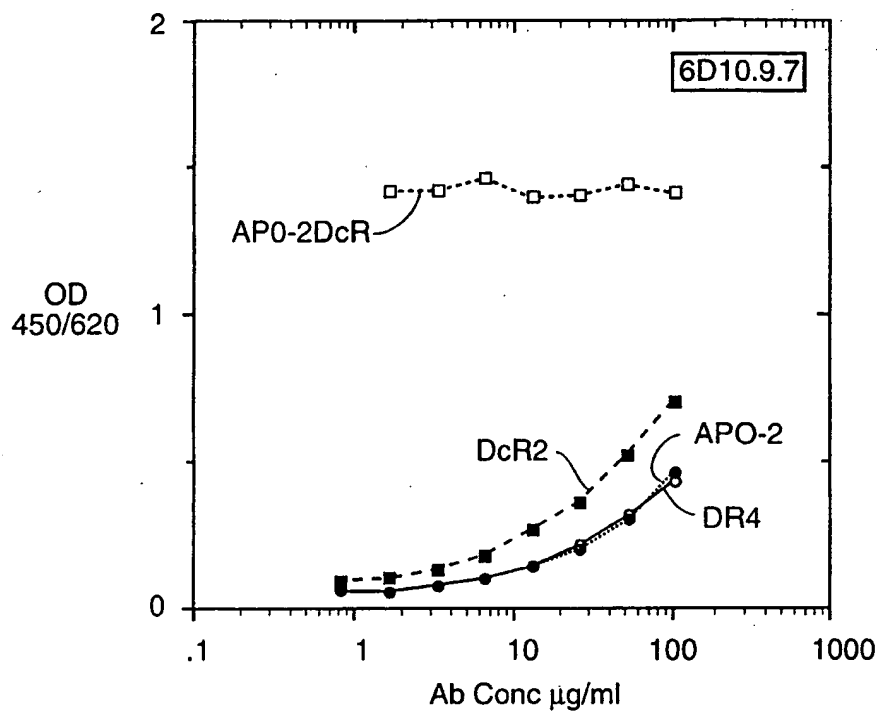
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**FIG. 14A****FIG. 14B****FIG. 14C**

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**FIG. 15A****FIG. 15B**

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**FIG. 15C**

## Summary of mAbs to DcR1

mAbs	ISOTYPE	FACS (HUMEC)	Cross reactivity			
			DR4	Apo-2	Apo-2DcR	DcR2
1C5.24.1	IgG1	+	++	+++	+++	-
4G3.9.9	IgG1	+	++	+	+++	+/-
6D10.9.7	IgG2b	+	-	-	+++	+/-

Percent Cross reactivity was determined by comparing the binding capacity to Apo-2DcR at 10 µg/ml of mAbs in ELISA. ++: >75%, +: 25-75%, +/-: 10-25%, -: <10%.

**FIG. 16**

# INTERNATIONAL SEARCH REPORT

Int .lional Application No

PCT/US 98/12456

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/715 A61K38/17 C12N15/62 G01N33/50  
C07K16/28 A01K67/027 C12N5/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	SHERIDAN J P ET AL: "CONTROL OF TRAIL-INDUCED APOPTOSIS BY A FAMILY OF SIGNALING AND DECOY RECEPTORS" SCIENCE, vol. 277, 8 August 1997, pages 818-821, XP002065023 see the whole document ---	1-17, 21, 35-40, 43, 44, 49-52
P, X	MACFARLAND M ET AL: "IDENTIFICATION AND MOLECULAR CLONING OF TWO NOVEL RECEPTORS FOR THE CYTOTOXIC LIGAND TRAIL" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 41, 10 October 1997, pages 25417-25420, XP002065148 see the whole document --- -/--	1-14, 35-40, 43, 44, 49, 51, 52

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

27 October 1998

Date of mailing of the international search report

05. 11. 98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Oderwald, H

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/12456

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>SCHNEIDER P ET AL: "CHARACTERIZATION OF TWO RECEPTORS FOR TRAIL" FEBS LETTERS, vol. 416, 27 October 1997, pages 329-334, XP002065022 see the whole document ---</p>	<p>1-11, 35-40, 43, 44, 49, 51, 52</p>
A	<p>CHINNAIYAN A M ET AL: "SIGNAL TRANSDUCTION BY DR3, A DEATH DOMAIN-CONTAINING RECEPTOR RELATED TO TNFR-1 AND CD95" SCIENCE, vol. 274, no. 5289, 8 November 1996, pages 990-992, XP000676685 see the whole document ---</p>	
A	<p>PITTI R M ET AL: "INDUCTION OF APOPTOSIS BY APO-2 LIGAND, A NEW MEMBER OF THE TUMOR NECROSIS FACTOR CYTOKINE FAMILY" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 22, 31 May 1996, pages 12687-12690, XP002065021 see the whole document -----</p>	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 98/12456

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 53 and 54 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 29-31  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
Claims 29-31 have not been searched due to insufficient disclosure of the claimed cell lines.
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.